UNIVERSIDADE FEDERAL DO PARANÁ

JEFFERSON DA LUZ COSTA

CALLUS GROWTH KINETICS OF PHYSIC NUT (*Jatropha curcas* L.) AND ANTHELMINTIC AND CYTOTOXIC ACTIVITY

CURITIBA

2016

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Tese apresentada como requisito parcial à obtenção do grau de Doutor do Programa de Pós - Graduação em Engenharia de Bioprocessos e Biotecnologia do Setor de Tecnologia da Universidade Federal do Paraná.

Orientador: Dr. Carlos Ricardo Soccol
Co-Orientador: André Luis Lopes da Silva

CURITIBA
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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pos-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **JEFFERSON DA LUZ COSTA**, intitulada "**CALLUS GROWTH KINETICS OF PHYSIC NUT (Jatropha curcas L) AND ANTHELMINTIC AND CYTOTOXIC ACTIVITY**", apos terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO

Curitiba, 29 de Dezembro de 2016

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TERMO DE APROVAÇÃO

JEFFERSON DA LUZ COSTA

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Tese aprovada como requisito parcial para obtenção do grau de Doutor no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, pela seguinte banca examinadora.

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DEDICATÓRIA

Aos meus pais, Raimundo e Amujaci por todo amor, incentivo e carinho dedicados durante minha vida. Aos meus irmãos Gustavo, Rayanne e Júnior, e minha sobrinha Heloisa Costa pelo seu apoio, paciência e amor incondicional!

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EPÍGRAFE

“Seja senhor das tuas vontades e escravo da tua consciência.”
(Aristóteles)

“O espírito de um verdadeiro guerreiro apenas se revela na hora de enfrentar as adversidades!“

“As pessoas costumam dizer que a motivação não dura muito. Um banho também não - por isso recomenda-se que os dois sejam diários.”
(Zig Ziglar)

RESUMO

A cultura de tecidos vegetais propicia em um período curto de tempo a multiplicação de plantas geneticamente idênticas em larga escala, permite a produção de metabólitos secundários em ambiente controlado para aplicação na agricultura e na indústria. Pinhão manso (*Jatropha curcas* L.) é uma planta usada para produção de biodiesel com alto teor de óleo e apresenta grande quantidade de compostos bioativos que podem ser utilizados para desenvolvimento de novos produtos na indústria. Por essa razão os objetivos desse trabalho foram: (1) Estabelecer curva de crescimento de calos e avaliar o perfil dos ácidos graxos do óleo bruto (pinhão manso); (2) Avaliar a atividade anti-helmíntica do extrato aquoso quente do grão e do calo de *J. curcas*, testar a toxicidade dos extratos em náuplios *de Artemia salina* e identificar os possíveis compostos bioativos associados à atividade anti-helmíntica. (1) A cinética da curva de crescimento de calos de pinhão manso apresentou padrão de curva sigmoidal com seis fases distintas: lag, exponencial, linear, desaceleração, estationária e de declínio. O perfil lipídico do óleo bruto dos calos apresentaram ácidos graxos de cadeias médias e cadeias longas, onde o ácido graxo de maior percentagem no extrato foi o ácido palmítico (55%) e o perfil lipídico do extrato do calo é diferente do perfil lipídico da semente de *J. curcas*. (2) Extrato aquoso quente do grão (pHs 5,7 e 9) e do calo (pH 5), 115 dias de cultivo, de *J. curcas* obtiveram atividade anti-helmítica e os extratos não foram citotóxicos, testados em *Artemia salina*. Os fitoquimicos nematicidas não foram completamente elucidados. Em diferentes pHs de extração e tipos de tecidos apresentou diferentes compostos nematicidas, como acidos graxos, furfural e ésteres de forbol.

Palavras-chave: Cultura de tecidos de plantas, composição lipídica, nematicida natural, ésteres de forbol, calogênese, análises *in silico*.

ABSTRACT

The plant tissue culture allows the multiplication of genetically identical plants on a large scale, allowing the production of secondary metabolites in a controlled environment for application in agriculture and industry. *Jatropha curcas* L. is a plant used for the production of biodiesel with high oil content and presents a great amount of bioactive compounds that can be used to develop new products in the industry. The purpose of the present study were: (1) To establish a calli growth curve and to evaluate the fatty acid profile of crude oil extracted from callus.; (2) To evaluate the anthelmintic activity of hot aqueous extracts of kernels and calli of *J. curcas*, the toxicity effects of these extracts against *Artemia salina* nauplii and to identify the bioactive compounds associated to anthelmintic activity. (1) The Kinetics of the callus growht curve presented a sigmoid standard curve with six distinct phases: lag, exponential, linear, deceleration, stationary and decline. The calli produced medium-chain and long-chain fatty acids and the palmitic acid was the fatty acid with the highest proportion in oil. The lipid profile obtained in callus oil was different from the seed oil profile. (2) The hot aqueous extracts of *J. curcas* kernel (pHs 5,7 and 9) and callus (pH 5), 115 days of culture was efficient for anthelmintic activity, in lethal concentration, 50%, (LC_{50}), and these extracts were not toxic against *Artemia salina.* The nematicidal fitochemicals were not completed elucidated. The different pH extraction and tissue type presented different nematicidal compounds, such as fatty acids, furfural and phorbol esters.

Keywords Plant Tissue culture • lipid composition • natural nematicide • phorbol esters • callogenesis • *in silico* analysis

SUMMARY

1 INTRODUCTION

Plant tissue culture techniques used in the culture by propagating elite clones of controlled conditions. The ability of morphogenesis is controlled by cell totipotency, can be directed to organogenesis or embryogenesis and to most varied applications: massive multiplication of selected plants, cell culture, genetic transformation, protoplast culture. Diverse approaches for metabolites production involving plant tissue culture can be performed, such as calli culture, cell suspension culture, hairy root and multiple shoots.

Physic nut (*Jatropha curcas* L.) belongs to Euphorbiaceae family, is native of the american tropics and cultivated in tropical and subtropical regions around the world and currently is in domestication process. It presents high oil content, drought tolerance, easy cultivation and its oil is used for biofuel production. Moreover, this plant is an important candidate for the development of new products such as biopesticides, antihelmintics, molluscicides and fungicides. The main base for these products are the phorbol esters, which have molluscicidal, insecticidal, fungicidal activities. Such properties have been demonstrated in lab-scale experiments. On the other hand, the compound responsible for the anthelmintic activity remains unknown.

The concentration and composition of plant secondary metabolites can be influenced by climate, soil, genetic factors, harvest time and solar radiation. It is known that the chemical composition of seeds of *J. Curcas* can vary in some regions. Moreover, the protocols and solvents used for extraction promote variation in concentrations of the metabolits present in extracts revealed the presence of bioactive constituents that may be responsible for the anthelmintic activity. Anthelmintics are drugs that may act locally to expel worms from the gastrointestinal tract (GIT) or systemically to eradicate adult helminths or development forms that invade organs and tissues (Jain and Jain, 1972). The World Health Organization (WHO) reveals that over two billion people are suffering from parasitic worm infections is estimated that by the year 2025, about 57% of the population in developing countries will be influenced (Mulla, et al., 2010).

The first chapter describes to establish a calli growth curve and to evaluate the fatty acid profile of crude oil extracted from callus of *Jatropha curcas* L. The physic nut (*Jatropha curcas* L.) is a perennial plant that belongs to Euphorbiaceae family and currently is in domestication process and this protocol represents a tool to candidate for

the development of new products such as biopesticides, antihelmintics, molluscicides and fungicides. The second chapter approaches to evaluate the anthelmintic activity of hot aqueous extracts of kernels and calli of *J. curcas*, the toxicity effects of these extracts against *Artemia salina* nauplii and to identify the bioactive compounds associated to anthelmintic activity.

1.1 General objective

In order to develop new nematicidal from *Jatropha curcas* L., such as to establish calli growth kinetics, evaluate the fatty acid profile of crude oil extracted from callus, to evaluate the anthelmintic activity of hot aqueous extracts of kernels and calli.

1.2 Specific objectives

1. In order to establish calli growth kinetics;

2. To evaluate the fatty acid profile of crude oil extracted from callus;

3. To evaluate the anthelmintic activity of hot aqueous extracts of kernels and calli of *J. curcas*;

4. To evaluate the toxicity effects of hot aqueous extracts of kernels and calli of *J. curcas* against *Artemia salina* nauplii;

5. To identify the bioactive compounds associated to anthelmintic activity.

CHAPTER I (RESEARCH RESULTS) - CALLUS GROWTH KINETICS OF PHYSIC NUT (*Jatropha curcas* L.) AND CONTENT OF FATTY ACIDS FROM CRUDE OIL OBTAINED *in vitro.*

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ABSTRACT

The callus growth kinetics allows identifying the appropriate moment for callus pealing and monitoring the accumulation of primary and secondary metabolites. The physic nut (*Jatropha curcas* L.) is a plant species used for biofuel production due to its high oil content; however this plant presents a great amount of bioactive compounds which can be useful for industry. The aim of this research was to establish a calli growth curve and to evaluate the fatty acid profile of crude oil extracted from callus. The callus growth kinetics presented a sigmoid standard curve with six distinct phases: lag, exponential, linear, deceleration, stationary and decline. Total soluble sugars were higher at the inoculation day. Reducing sugars were higher at the inoculation day and at the 80th day. The highest percentage of ethereal extract (oil content) was obtained at the 120th day of culture, reaching 18% of crude oil from the callus. The calli produced medium-chain and long-chain fatty acids (from 10 to 18 carbon atoms).The palmitic acid was the fatty acid with the highest proportion in oil (55.4%). The lipid profile obtained in callus oil was different from the seed oil profile.

Keywords Lipid profile • callus curve • Lipid composition • Callogenesis • Tissue culture • Primary metabolites

1 INTRODUCTION

The physic nut (*Jatropha curcas* L.) is a perennial plant that belongs to Euphorbiaceae family and currently is in domestication process (Costa et al. 2011;

Horbach et al. 2014). It presents high oil content, drought tolerance, easy cultivation and its oil is used for biofuel production. Moreover, this plant is an important candidate for the development of new products such as biopesticides, antihelmintics, molluscicides and fungicides. The main base for these products are the phorbol esters, which have molluscicidal (Goel et al. 2007; Zhang 2012; Devappa et al. 2012), insecticidal (Li et al. 2004) and fungicidal activities (Devappa et al. 2012). Such propertieshave been demonstrated in lab-scale experiments. On the other hand, the compound responsible for the anthelmintic activity remains unknown. However, other compounds were also identified for molluscicidal activity, such as Curcusone D and Jatropholone B (Zhang 2012).

Several compounds present in the physic nut have a great potential to be used for the development of new pharmaceutical drugs. Noteworthy, for cancer disease, some compounds already identified which present antitumor activities, as the jatrophone (Kupchan et al. 1976), curcacycline A (Van der Berg et al. 1995), curcin (Lin et al. 2003) jatrophine, jatropham and curcain (Reddy and Pamidimarri, 2010). Moreover, this species presents also antimicrobial activities, as well as for some fungi (Devappa et al. 2012). Curcin at 5 $\mu g.mL^{-1}$ inhibited hyphal growth and spore formation in *Pyricularia oryzae*, *Pestalotia funereal*, *Rhizoctoniasolani*and*Sclerotiniasclerotiorum* (Wei et al. 2004). The seed oil can be applied to treat eczema and skin diseases and to soothe rheumatic pain (Heller 1996).

The plant tissue culture is an important tool that allows the production of primary and secondary metabolites in environmental controlled conditions, independent of climatic conditions. Diverse approaches for metabolites production involving plant tissue culture can be performed, such as calli culture, cell suspension culture, hairy root and multiple shoots. Moreover, the culture in bioreactors can promote a higher production of biomass than the conventional culture using flasks (Scheidt et al. 2011). Calli induced from endosperm of physic nut can be used to produce curcin (Rong and Wang 2005). However, the knowledge of the metabolite production and its biosynthesis in physic nut calli is insufficient. Nevertheless, the production of oils from callus can be an interesting strategy to study the biosynthesis of fatty acids and other metabolites including industrial applications.

The aim of this research was to establish calli growth kinetics and to evaluate the fatty acid profile of crude oil extracted from callus.

2 Material and methods

2.1 Plant material and culture conditions

Seeds of a toxic variety of physic nut (*Jatropha curcas* L.) were purchased from Carol Company, Dourados City, Mato Grosso do Sul State, Brazil. In order to establish the seedlings (explant donors)*in vitro*, seeds were decoated and immersed in 70% ethanol for 1 min and immersed in 2.5% sodium hypochlorite for 10 min (all solutions were agitated manually in laminar flow cabinet during seed immersion), after the disinfection the seeds were washed four times with distilled water and autoclaved. The seeds were sowed on MS medium (Murashige and Skoog, 1962) with 30 $g.L^{-1}$ sucrose and solidified with 6 $g.L^{-1}$ agar (Vetec^{TM}). The pH was adjusted to 5.8 (Costa et al. 2010). All explants were maintained in a growth chamber under light (photoperiod of 16 h), at a temperature of 25 ± 2°C and under a light intensity of 40 $\mu M.m^{-2}.s^{-1}$ produced by white fluorescent lamps.

2.2 Callus kinetics growth

In order to induce calli formation, hypocotyl explants were excised from seedlings, and after 15 days they germinated with approximately 1.0 cm length. The inoculation of explants was carried out on MS medium containing 3% sucrose, 0.7% agar, pH adjusted to 5.8 and supplemented with 0.5 $mg.L^{-1}$ 2,4-D (2,4-Dichlorophenoxyacetic acid) (Soomro and Memon 2007). The hypocotyl weight was discounted from the calli weight. There was no subculture during the experimental period. Fresh weight, dry weight, total soluble sugars, reducing sugars, total protein and amino acids were analyzed each 10 days after the inoculation of the explants, until the 180^{th} day.

2.3 Biochemical analysis

In order to prepare the analysis, three samples containing 500 mg calli were homogenized in a grail with 5 mL 0.1 M potassium phosphate buffer (pH 7.5). The total protein content and amino acids were determined using the methodology described by Bradford (1976) and Stein and Moore (1948), respectively. The levels of soluble sugars

were determined using the method described by Yemm and Willis (1954). The reducing sugars were determined using the methodology described by Miller (1959). In order to determine the ethereal extract from callus tissue, the callus oil was extracted using petroleum ether (55-60^{o} C) in a soxhlet extractor for 5h at room temperature.

2.4 Analysis of fatty acid composition

The methyl esters were prepared from the total lipids (crude oil from callus tissues) by the method of AOAC (1990). These fatty acid methyl esters were analyzed by a gas chromatography coupled with a mass spectrometry detector (GC-MS, Varian brand), model 3800 CP/Saturn 2000 equipped with the CP-Sil CB 8 column (30 m X 0.25 mm). The initial temperature was 60º C with an elevation rate of 3° C/min until 250 ºC. The split was 1/200. The scanning range was from 30 to 500 m/z. The identification was carried out in comparison with the NIST 98 MS Library (Varian 1998) in addition to the specific literature (Adams 2007). The linear retention index and the theoretical retention confirmed the identifications. A standard fatty acid methyl ester mixture was run and the retention times were used to identify the sample peaks. Fatty acid levels were estimated as area percent of total peak area of methyl esters.

2.5 Statistical analysis

The experimental design was a completely randomized design with three replicates; ten test tubes were used per replicate. The data was submitted in a normality analysis for the Lilliefors´s test, and submitted to the analysis of variance (ANOVA) followed by regression analysis. All statistical analyses were done following the procedures of the software SOC (Embrapa 1990).

3 Results and discussion

3.1 Calli growth kinetics

The calli originated in this experiment had a compact appearance, and no visual changes in its appearance were observed until the end of the experiment. The growth curve determined by the observed points (i.e. the curve plotted using points obtained by

measurements), for the dry and fresh calli presented a sigmoid standard curve with six distinct phases: lag, exponential, linear, deceleration, stationary and decline (Figure 1). The lag phase occurred until 10 days after the experiment installation, during this period no morphogenic event was visually verified. The exponential phase began at the 10thday and finished at the 60th day. During this period calli induced from hypocotyls began to proliferate. The linear phase occurred between the 60th and the 100th days. During this period an intense cellular proliferation was observed resulting in a fast growth of calli. The deceleration phase occurred after the 100th day and finished at the 120th day, when the higher yield of callus biomass was obtained, an average of 93.9 g. The stationary phase was verified from the 120th day until the 130th day, followed by a decline phase that began at the 130th day (Figure 1). The most suitable mathematical model to describe the data was the logistic model, used to estimate both fresh and dry calli growth kinetics. Both the models presented a determination coefficient of 0.98 (Figure 1).

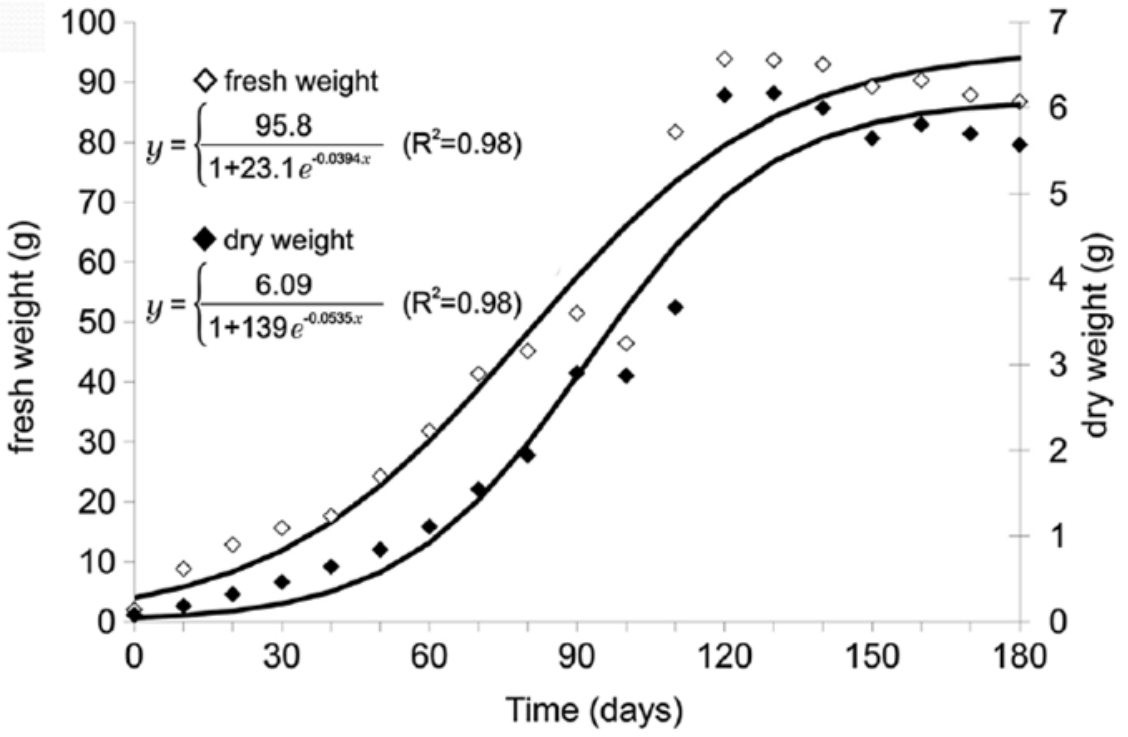

Figure. 1 Callus growth kinetics originated from hypocotyls of physic nut (*Jatropha curcas* L.) cultivated on MS medium supplemented with 0.5 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) until 180 days of *in vitro* culture. Dry and fresh weight of callus.

The calli growth kinetics is important to decide the management of calli culture, mainly to decide when these calli must be pealed and to determine when a great amount of secondary metabolites will be produced. According to Smith (1992) in the deceleration phase (in our study it occurred between the 100th and the 120th days) it is

recommended the calli pealing for another new culture medium due to nutrient reduction, loss of water from culture medium and toxic substances accumulation. The ideal period for extraction of secondary metabolites is the stationary phase (in our study it occurred between the 120th and the 140th days), when the production of the primary metabolites practically ceases and the accumulation of secondary metabolites is beginning. However, studies of different secondary metabolites must be carried out to establish their kinetics and to evaluate the factors involved in their production. The dry calli growth kinetics is important for the management involving industrial applications, whereas dry biomass is more stable than the fresh biomass, moreover it is necessary to know the dry biomass yield because it is used in industrial process. The largest yield of dry calli biomass had an average of 6.2 g at the 120th day of culture, representing 6.6% of the original fresh biomass of callus.

3.2 Total protein and amino acids

In order to adjust the regression equations for total protein content from callus tissue, two models were adjusted to explain different situations from different periods of kinetics. The best model to describe the data from the installation of the experiment to the 79th day was the exponential model and from the 80th to the 180ththe quadratic model was the best one. The determination coefficient was 0.92 referring to all curve points (Figure 2).

During the lag phase (until the 10th day), the total protein accumulation was extremely low and its increment was negligible. But increased considerably at exponential phase until the 80th day, in which occurred the largest production of total proteins in callus tissue and resulted in 0.15 μg.g^{-1}calli fresh weight, which corresponded to linear phase, characterized by active cellular growth. After the 80th day the total protein accumulation started to decrease progressively in successive phases (deceleration, stationary and decline). The lower value obtained by total protein was observed at the 160th day and continued stable until the end of the experiment (Figure 2).

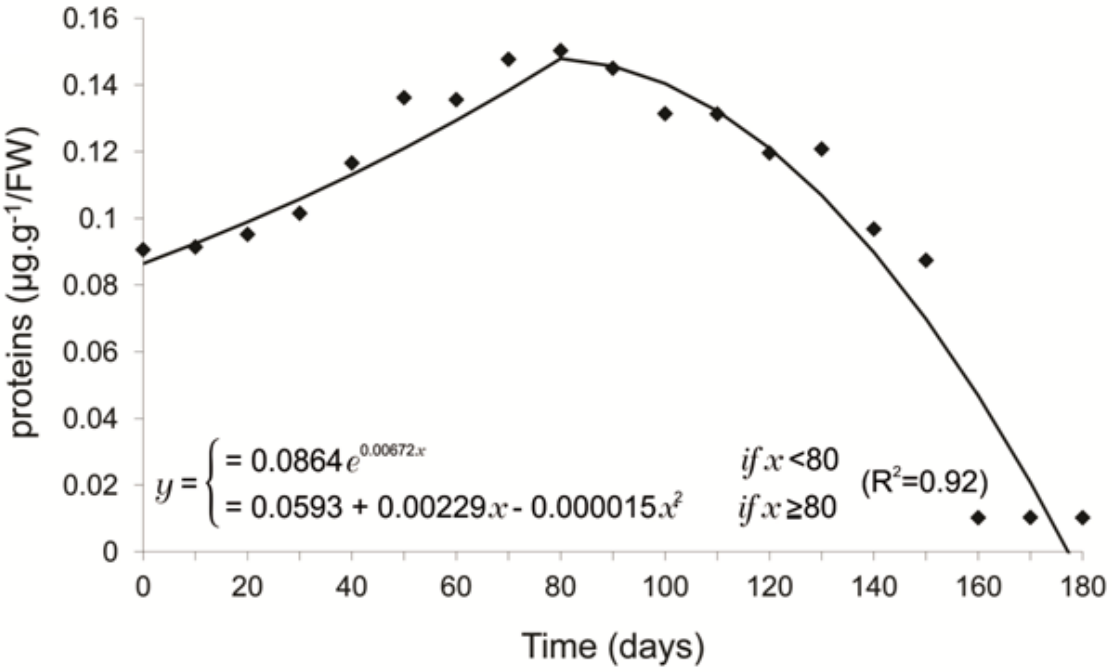

Figure. 2 Callus growth kinetics originated from hypocotyls of physic nut (*Jatropha curcas* L.) cultivated on MS medium supplemented with 0.5 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) until 180 days of *in vitro* culture. Total protein content from callus tissues.

The increment in protein content suggests changes in cellular metabolism, mainly due to the necessity of specific proteins to perform different biological functions; also, many enzymes (proteins) must be produced to accompany the cellular growth and its maintenance, moreover, many of these proteins play structural roles in the cells. When the callus becomes mature (i.e. the primary growth is almost complete), next to the deceleration phase, the protein content start to decrease, mainly because some proteins can be used as energy source, whereas the carbon source (i.e. sucrose) of the culture medium can be almost exhausted at this point.

In order to adjust the equations for the kinetics of total amino acid content from callus tissue, two models were adjusted to explain the different periods. The cubic model presented the best adjustment from the installation of the experiment to the89th day, while the multiple multiplicative factor model presented the best adjustment from the 90thto the 180th day. The determination coefficient was 0.89 referring to all curve points (Figure 3).

During the lag phase and the beginning of exponential phase (until the 20th day) there was a great increment in total amino acid content from callus tissue (Figure 3). This great and fast amino acids accumulation can be associated with the efficient use of the nitrogen sources presents in culture medium. From the 20th to the 60th day there was a

relative stability which corresponds to the exponential phase, and it is hypothesized that the amino acids have been used in biosynthesis of proteins, whereas in this phase there was an increment in protein accumulation in callus tissue (Figure 2). There was an increase in amino acids content from the 60thto the 90th day. Almost at the end of linear phase, the amino acid content began to decrease until the end of the experiment (Figure 3). The decrease of amino acid content coincided with the beginning of deceleration phase and followed decreasing in successive phases and it is hypothesized that the amino acids have been used as nitrogen source, similarly to what happened with proteins, due to shortage of the nitrogen source of culture medium.

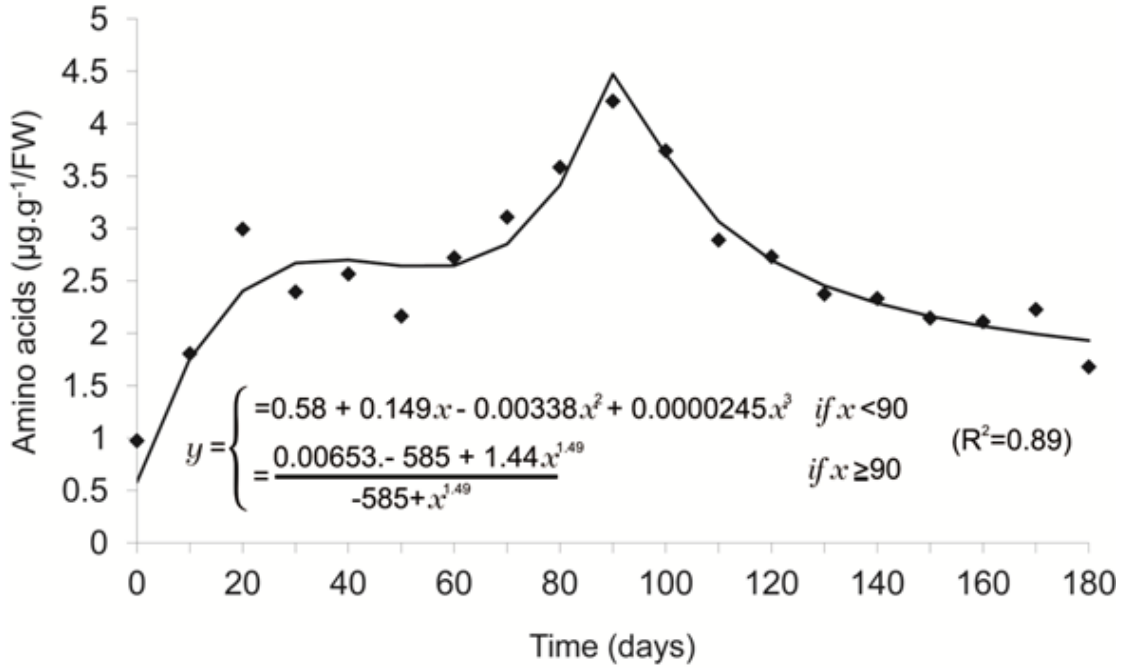

Figure. 3 Callus growth kinetics originated from hypocotyls of physic nut (*Jatropha curcas* L.) cultivated on MS medium supplemented with 0.5 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) until 180 days of *in vitro* culture. Amino acid content from callus tissues.

The lowest value for total amino acids was 0.91 µg per gram of calli fresh weight, and occurred at the beginning period of the culture, this reflect the nutritional status of the explant (hypocotyls). Different results were obtained in *Byrsonima intermedia*, in which the initial explants (i.e. leaf explants) at the start of culture presented the highest content of amino acids, what was possibly caused by the nutrition state of the explant before the excision (Nogueira et al. 2008).

3.3 Total soluble sugars and reducing sugars

The most suitable mathematical model to describe the data for the total soluble sugars kinetics was the linear model. The determination coefficient was 0.87 (Figure 4). Total soluble sugars were higher at the installation of the experiment and decreased linearly until the end of the experiment. Similar results were found in *Bertholletia excelsa* and in *Garcinia bralisiensis* in which the total soluble sugars were higher on the inoculation day and decreased during the growth kinetics (Serra et al. 2000; Santos-Filho et al. 2014). Higher sugar contents at the inoculation day can be associated to the energetic reserves of the explant and were consumed to supply the energetic necessity of all metabolic processes of the calli. This result suggests a difficulty of the callus uptakes the sucrose presents in culture medium.

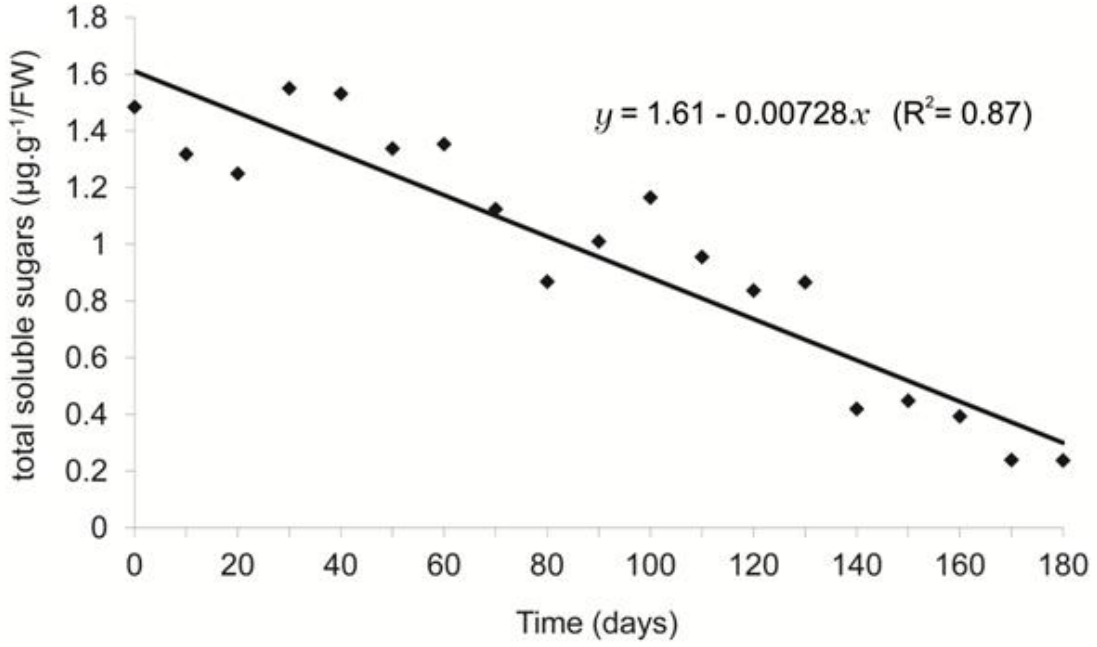

Figure 4. Callus growth kinetics originated from hypocotyls of physic nut (*Jatropha curcas* L.) cultivated on MS medium supplemented with 0.5 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) until 180 days of *in vitro* culture. (A) Total soluble sugars.

In order to adjust the regression equations for the kinetics of consumption of reducing sugars for callus tissue growth, two models were adjusted to explain different situations in different periods. The best model adjusted from the installation of the experiment to the 79th day was the rational model and from the 80th day to the 180th day the best one was the multiple multiplicative factor model. The determination coefficient was 0.99 referring to all curve points (Figure 5). Reducing sugars were higher at the inoculation day and at the 80th day (i.e. corresponding to linear phase) and then decreased until the end of the experiment (Figure 5).

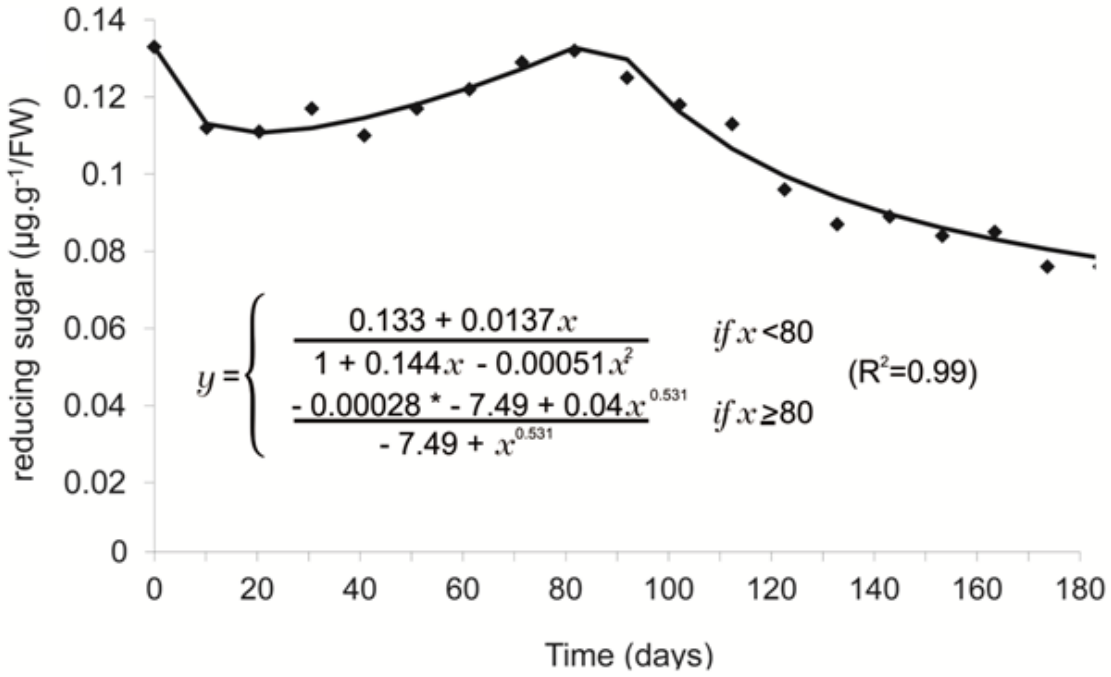

Figure 5. Callus growth kinetics originated from hypocotyls of physic nut (*Jatropha curcas* L.) cultivated on MS medium supplemented with 0.5 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) until 180 days of *in vitro* culture. Reducing sugars from callus tissues.

Reducing sugars are the first sugars consumed in the cellular metabolism due its higher reactivity than the non-reducing sugars (Carvalho et al. 2013). The higher level of reducing sugars at the initial phase of the culture can be associated to the nutritional status of the initial explant (i.e. hypocotyl) and the second peak with higher level of reducing sugars that occurred at 80th day can be attributed to the glucose and fructose uptake originated from cleavage of sucrose presents in culture medium. The cleavage of sucrose in monosaccharides could have occurred due to instability in the culture medium influenced by putative pH changes or by explant exudation of putative compounds which could promote the sucrose cleavage.

3.4 Ethereal extract

The highest percentage of ethereal extract (oil content) was obtained at the 120th day of culture, reaching 18% of crude oil from the callus. For this characteristic, two mathematical models were proposed: one explaining the crude oil accumulation inside the callus tissues and the other explaining the oil consumption by the callus tissue. For oil accumulation a quadratic model was adjusted and for the oil consumption a rational model was adjusted (Figure 6).

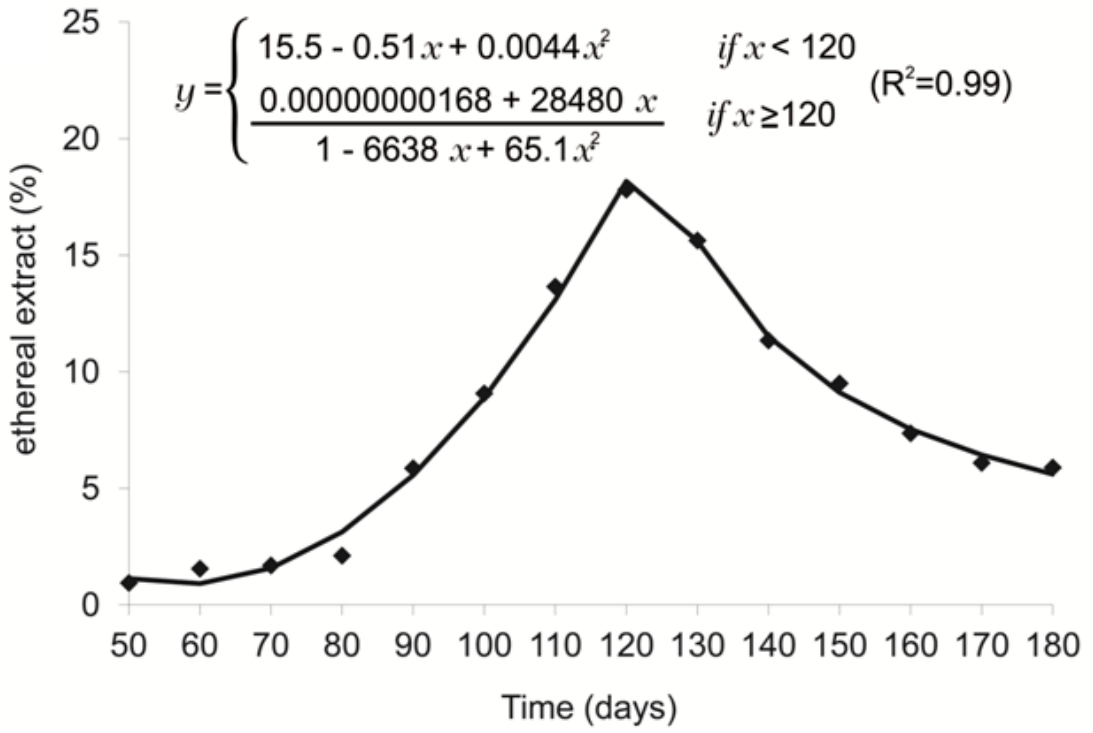

Figure 6. Callus growth kinetics originated from hypocotyls of physic nut (*Jatropha curcas* L.) cultivated on MS medium supplemented with 0.5 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) until 180 days of *in vitro* culture. Ethereal extract (crude oil) from callus tissues.

An oil exudation could also be observed from callus tissues to the culture medium, suggesting that the amount of oil produced can be higher than 18%. It is already known that oil plants need also fatty acids as carbon sources, as observed in *Cocos nucifera* embryos cultivated *in vitro* on lauric acid, wherein the lauric acid stimulated the plantlet growth and its development (López-Villalobos et al. 2011). The oil yield in the calli was lower than the amount produced in seeds, whereas the oil content of seed kernel from 11 counties varied from 51.3% to 61.2% (Li et al. 2006). Nevertheless, *in vitro* it is possible to produce oil throughout the year, while the physic nut plants produce seeds once a year. In order to produce fatty acids from callus tissues, the calli must be harvest around the 120th day of *in vitro* culture.

3.5 Fatty acid composition

The calli produced medium-chain and long-chain fatty acids (from 10 to 18 carbon atoms) (Table 1). The medium-chain fatty acids were saturated (i.e. capric acid and lauric acid) while the medium-chain fatty acids, produced in higher amounts (93.76%), were saturated and unsaturated. The percentage of the unsaturated and

saturated fatty acids was of 9.92% and 90.08%, respectively. The oleic, stearic and linoleic acids (C18) represented 13.77% of the oil. The palmitic acid was the fatty acid with the highest proportion in oil (55.4%), followed by pentadecanoic acid (11.63%) and myristic acid (11.25%) (Table 1). The palmitic acid was also the fatty acid found in higher concentration in calliof melon induced by different tissues such as hypocotyls, cotyledons, roots, leaves and stems (Halder and Gadgil, 1984).

Table 01. Determination of the fatty acid composition of crude oil of physic nut (*Jatropha curcas* L.) callus after 110 days of *in vitro* growth by gas chromatography-mass spectrometry (GC-MS). Extraction was performed by the Soxhlet Method using petroleum ether (ethereal extract).

				$mg.L^{-1}$	wt%	
Structure	Formula	Fatty acid	Systematic name	Callus oil	Callus oil	Seed oil[a]
C10:0	$C_{10}H_{20}O_2$	Capric acid	Decanoic acid	9.178	2.52	-
C12:0	$C_{12}H_{24}O_2$	Lauric acid	Dodecanoic acid	13.358	3.72	-
C14:0	$C_{14}H_{28}O_2$	Myristic acid	Tetradecanoic acid	47.031	11.25	0.15-1.18
C15:1	$C_{15}H_{28}O_2$	Pentadecenoic Acid	CIS-10-Pentadecenoic Acid	21.856	1.71	-
C15:0	$C_{15}H_{30}O_2$	Pentadecylic acid	Pentadecanoic acid	27.003	11.63	-
C16:0	$C_{16}H_{30}O_2$	Palmitic acid	Hexadecanoic acid	232.651	55.40	10.5-13.0
C18:1	$C_{18}H_{34}O_2$	Oleic acid	(9Z)-Octadec-9-enoic acid	31.204	7.43	2.27-2.80
C18:0	$C_{18}H_{36}O_2$	Stearic acid	Octadecanoic acid	23.345	5.56	41.5-48.8
C18:2	$C_{18}H_{32}O_2$	Linoleic acid	(9Z,12Z)-9,12-Octadecadienoic acid	2.864	0.78	34.6-44.4
C18:3	$C_{18}H_{30}O_2$	α-Linolenic acid	(9Z,12Z,15Z)-9,12,15-Octadecatrienoic acid	-	-	0.11-0.21
C20:1	$C_{20}H_{38}O_2$	Cis-11-Eicosenoic acid	(Z)-Eicos-11-enoic acid	-	-	0.12-0.19
C20:2	$C_{20}H_{36}O_2$	Cis-11,14-Eicosenoic acid	(2E,4E)-2,4-Icosadienoic acid	-	-	0.1-0.13
Total				408.490	100	

In four provenances of Mexico the major fatty acids found in the oil samples were oleic (41.5–48.8%), linoleic (34.6–44.4%), palmitic (10.5–13.0%) and stearic (2.3–2.8%) acids (Martínez-Herrera et al., 2006).

The lipid profile obtained in callus oil was different from the seed oil profile (Table 1). There are many differences in the oil produced in callus, such as the presence of fatty acids (capric, lauric, pentadecenoic and pentadecylic) that are not present in seed oil. The callus oil had myristic acid, palmitic acid and oleic acid in larger proportions than seed oil. The seed oil presents higher proportions of stearic acid and linoleic acid than callus oil (Table 1). These differences in lipid profile of callus and seed oil are probably due to differences in the gene expression of cells of different

tissues (calliand embryos), which results in changes in the metabolism of these cells and tissues.

Similar results were found for the lipid profile obtained from cell suspension of physic nut which was different from the seed oil profile; however there was a great variation on the lipid profile among different populations of physic nut, as well as qualitatively and quantitatively in these cell suspensions, being difficult to establish a profile for oil produced from cell suspension (Correa and Atehortúa 2012). Consequently, it is possible that calli obtained from different populations of physic nut could present different lipid profile or other metabolites. In this case, appropriate genotypes must be selected according to the desired metabolite. However, the callus growth kinetics in the present study can be useful to carry out these researches.

4 Conclusions

The callus growth kinetics presented a sigmoid standard curve with six distinct phases: lag (0-10th days), exponential (10-60th days), linear (60-100th days), deceleration (100-120th days), stationary (120-130th days) and decline (after the 130th day). The highest percentage of ethereal extract (oil content) was obtained at the 120th day of culture, reaching 18% of crude oil from the callus. The calli produced medium-chain and long-chain fatty acids (from 10 to 18 carbon atoms).The lipid profile obtained in callus oil was different from the seed oil profile. The palmitic acid was the fatty acid with the highest proportion in oil (55.4%). The callu oils presented some fatty acids (i.e. capric, lauric, pentadecenoic and pentadecylic) that are not present in seed oil. The callus oil had myristic acid, palmitic acid and oleic acid in larger proportions than seed oil.

CHAPTER II (RESEARCH RESULTS) - ANTHELMINTIC ACTIVITY AND CYTOXICITY EFFECTS OF HOT AQUEOUS EXTRACTS OF KERNELS AND CALLUS OF PHYSIC NUT (*Jatropha curcas* L.) – Euphorbiaceae

Manuscript submitted for publication in the Journal Of Ethnopharmacology

ABSTRACT

Studies were undertaken on the nematicidal activity of hot aqueous extracts in different pHs of *Jatropha curcas* kernel against *Caenorhabditis elegans*. The most effective pH extract was 5, followed of 7 and 9, presenting LC_{50} (lethal concentration, 50%) of 16.76, 24.02 and 30.02 $mg.mL^{-1}$. Hot aqueous extract (pH 5) obtained from callus tissue (with 115 days of culture) also has nematicidal activity with LC_{50} of 14.52 $mg.mL^{-1}$. These extracts were not toxic against *Artemia salina*. Biocompounds screening was perfomed using different analytical approaches. The biological activity of the compounds found in the hot aqueous extracts, evaluated by *in silico* analysis through prediction of activity spectra for substances (PASS) software, indicated that thirteen phytochemicals were expected to show anthelmintic activity at various levels. *In silico* studies showed that the values of Pa (probability to be active) for these biocompounds found and they varied from 0.436 and 0.865, respectively. The nematicidal fitochemicals were not completed elucidated, but different pH extraction and tissue type presented different nematicidal compounds, such as fatty acids, furfural and phorbol esters.

Keywords: *Artemia salina*, *Caenorhabditis elegans*, nematicidal activity, natural nematicide, phorbol esters, callus, *in silico* analysis.

INTRODUCTION

The physic nut (*Jatropha curcas* L.) belongs to Euphorbiaceae family and is an evergreen shrub. This species has gained special attention due to possess easy adaptation to diverse edaphoclimatic conditions and drought tolerance. Although this species is in domestication process (Costa et al., 2011; Horbach et al., 2014). It presents

high oil content, mainly designated to biodiesel production. Moreover, this plant is an important candidate for the development of new products such as biopesticides, anthelmintics, molluscicides, and fungicides (Costa et al., 2015).

The anthelmintic activity of the physic nut is already known traditionally. In Africa, the seeds are used as an anthelmintic (Watt and Breyer-Brandwijk, 1962) and the root decoction is drunk as vermifuge in Eastern Tanzania (Chhabra et al., 1990). However, some scientific studies have proven the nematicidal properties of the physic nut. Nematicidal properties were found in the seed oil (Shanker and Dhyani, 2006), in seed extract (Joymati et al., 1998; Eguale and Giday, 2009; Monteiro et al., 2011; Salles et al., 2014), in leaf extracts (Joymati et al., 1998; Suharti et al., 2010), in stem (Joymati et al., 1998) and in cake (Kalaiarasan et al., 2007). However, the bioactive compound or compounds with anthelmintic activity is yet unknown.

There is a great necessity to find new anthelmintic drugs to control the plant and animal-parasitic nematodes that have acquired resistance against the commercial nematicides. However, there is not prevision of new nematicide compounds for the next years, being necessary extensive studies to resolve this problem.

J. curcas has just a crop per year, this limit the use of its seeds to produce phytotherapic anthelmintics or nematicide formulations to be applied in soil. On the other hand, tissues produced *in vitro* in great scale could supplement a great amount of raw material to industry, avoiding the problems such as year station, pests, plant diseases and abiotic stresses on the plantations, which can influence in the metabolic compounds and result in variation of efficacy of phytotherapic or crude extracts. The plant tissue culture cans multiplicated genetically identical copies of a selected genotype in great amount in a short period of time (Silva et al., 2014). Moreover, the culture in bioreactors can promote a higher production of biomass than the conventional culture using flasks (Scheidt et al. 2011) or using hairy root culture (Makhzoum et al., 2013; Habibi et al., 2016).

Therefore, the aims of this study were to evaluate the anthelmintic activity of hot aqueous extracts of kernels and calli of *J. curcas*, the toxicity effects of these extracts against *Artemia salina* nauplii. Moreover, to identify the bioactive compounds associated to anthelmintic activity.

2 Material and methods

2.1 Chemical reagents

The chemical reagents 12-O-Tetradecanoylphorbol 13-acetate (TPA), cellobiose, glucose, xylose, xylitol, formic acid, acetic acid, ethanol, furfural, glucuronic acid, galacturonic acid, gluconic acid, mannose, arabinose, furfurilic acid, glycerol, levulinic acid and hydroxymethylfurfural were purchased from Sigma-Aldrich®, all reagents have ≥97% purity.

2.2 Plant material and culture conditions

Seeds of a toxic variety of physic nut (Jatropha curcas L.) were purchased from Carol Company, Dourados City, Mato Grosso do Sul State, Brazil. In order to establish the seedlings (explant donors) in vitro, seeds were decoated and immersed in 70% ethanol for 1 min and immersed in 2.5% sodium hypochlorite for 10 min (all solutions were agitated manually in laminar flow cabinet during seed immersion), after the disinfection the seeds were washed four times with distilled water and autoclaved. The seeds were sowed on MS medium (Murashige and Skoog, 1962) with 30 $g.L^{-1}$ sucrose and solidified with 6 $g.L^{-1}$ agar (Vetec™). The pH was adjusted to 5.8 (Costa et al. 2010). All explants were maintained in a growth chamber under light (photoperiod of 16 h), at a temperature of 25 ± 2°C and under a light intensity of 40 $\mu M.m^{-2}.s^{-1}$ produced by white fluorescent lamps.

2.3 Callus induction

In order to induce calli formation, hypocotyl explants were excised from seedlings (15 days old). The inoculation of explants was carried out on MS medium containing 30 $g.L^{-1}$ sucrose, 7 $g.L^{-1}$ agar, pH adjusted to 5.8 and supplemented with 0.5 $mg.L^{-1}$ 2,4-D (2,4-Dichlorophenoxyacetic acid) (Soomro and Memon, 2007).

2.4 Hot aqueous extraction

In order to obtain the extracts in different pHs, different distilled water volumes had its pH adjusted to pH 5, 7 and 9. Extracts were performed using callus tissue (pH 5) and kernels (pH 5, 7 and 9). Callus or kernel fresh weight (10 g) were macerated and

extracted in 100 mL boiled distilled water (1:10 w/v) during 30 min on water bath at 95^{o} C. After, the extracts remained at room temperature (25^{o} C) under 80 rpm agitation (in the dark) during 24 h, after they were filtered with Whatman No. 1 filter paper. The extracts were lyophilized. In order to determine fatty acids of the extracts, the total lipids were separated from samples by method of Bligh and Dyer (Bligh and Dyer, 1959).

2.5 Anthelmintic activity

C. elegans wild-type strain N2 (Bristol) and *E. coli* strain OP50 were generous gifts from Dr. Marcelo Mori (Federal University of Sao Paulo, Brazil). The stocks were maintained at 20 ˚C on *Escherichia coli* OP50/NGM (nematode growth media) plates as previously described (Brenner, 1974). Synchronous L1 populations were obtained by isolating embryos from gravid hermaphrodites using bleaching solution according to standard procedures (Solis and Petrascheck, 2011).

Assays for lethality with wild-type *C. elegans* were performed with complete S-medium in 96-well cell culture plates (Solis and Petrascheck, 2011). On the day of experiments, appropriate aliquots of the lyophilized extracts or 12-O-Tetradecanoylphorbol 13-acetate (TPA) stock solutions were added to S-medium containing 20 synchronized second-stage larvae (L2). Final concentrations of the lyophilized extracts were added in the range of 5.0–80.0 mg.mL^{-1}. Final concentrations of the 12-O-Tetradecanoylphorbol 13-acetate (TPA) were added in the range of 0.25–1.0 mg.mL^{-1}. As negative control experiment, nematodes were exposed to complete S-medium (free of any extract or drug). The nematodes were incubated for 24 h at 20 ˚C and the LC_{50} values, representing 50% lethality at a given concentration and 95% confidence interval (CI_{95}) were estimated. All assays were performed in triplicate.

2.6 Cytotoxicity of the extracts by *Artemia salina* bioassay

An adapted Solis et al. (1993) method by Gollo et al. (2016) was employed for the cytoxicity analysis of the extracts. A positive control was made from potassium dichromate at 0, 1, 3.125, 6.25, 12.5, 25 and 50 μg.mL^{-1}. Lyophilized powder of kernel and callus extracts (pH 5) were evaluated at the levels of 62.5, 125, 250, 500 and 1000 μg.mL^{-1}. After 24 h of treatment, the numbers of dead and living nauplii were counted,

and these data were used to estimate the LC_{50} (lethal concentration, 50%) and 95% confidence interval (CI_{95}). All assays were performed in triplicate.

2.7 Characterization by Fourier transform infrared spectroscopy (FTIR)**.**

The functional groups present in the samples of hot aqueous extracts of the kernels (pH 5, 7 and 9) and callus (pH 5) were determined by Fourier transform infrared spectroscopy (FTIR), performed in equipment VERTEX 70 (Bruker) equipment, using the DRIFT accessory (diffuse reflectance) with 64 scans, 4 cm^{-1} resolution, in the presense of atmospheric compensation in the region from 4000 to 400 cm^{-1}. Lyophilized extracts were used as samples. In order to determine the samples, approximatelly 20 mg were mixed with 100 mg KBr spectroscopic grade, homogenized and places on the DRIFT accessories and the spectra reported.

2.8 Analysis of carbohydrates and its derivatives

Cellobiose, glucose, xylose, xylitol, formic acid, acetic acid, ethanol, furfural, glucuronic acid, galacturonic acid, gluconic acid, mannose, arabinose, furfurilic acid, glycerol, levulinic acid and hydroxymethylfurfural were determined by High-Performance Liquid Chromatography (HPLC), in a Shimadzu Chromatograph equipped with an Aminex HPX-87H column, working in a furnace at 60^{o} C. Mobile phase was sulfuric acid (5 mM) at rate of 0.6 $mL.min^{-1}$ with an IR detector.

2.9 Phorbol esters (PHes) determination

Phorbol esters were extracted from samples after induction using 2 ml of methanol as co-solvent. After co-solvency of the reaction medium, the methanol was further used to determine the PHes concentration by Varian 320 LC-MS with MS mode for confirmation. PHes concentration was determined an aliquot was loaded on HPLC Varian 212 with reverse phase C18 LiChrophere 100, 5 µm (250 × 4 mm ID. from Merck) column. The column was protected with a head column containing the same material. The separation was performed at 45 °C temperature and the flow rate was 0.2 ml/min using isocratic elution, the mobile phase consisted of 1:1 (v/v) (88% ultrapure water/ 11% acetic acid/ 1% acetonitrile) with metanol. The injection volume was 20 µl

MS/MS conditions. The parameters used for the mass spectrometry under ESI+ mode were as follows: scan range, 50–1.000 m/z, multiple reactions monitoring (MRM) was used for the sensitive and selective detection of PHes, and other derivatives, capillary voltage 5.00 kV, shield potencial 600 V, detector EDR mode, cone voltage 120 V, source block temperature 55 °C, drying gas 20 psi, nebulizing gas 45 psi, API housing 55^{o} C, drying gas 180^{o} C. To achieve sensitive and selective detection of PHes and derivatives by LC–MS/MS, was identify the basic fragment ion shared by PHes and derivatives and also to optimize the MS/MS conditions accordingly. Next, in order to confirm whether the specified fragment ion was detected the PHes MS spectra were analyzed using the concentrated solutions. The detected peaks were qualitatively analyzed by comparing the MS/MS spectra of the fragments and identified by product ion scanning with PHes. The principal precursor ion (m/z = 311) originating in the frame unit and the product ion (m/z = 165; maximum ion intensity) were used for MRM. The detection limit of was 0.002 μg/mL in the MRM mode. The group of PHes peaks was detected at and appeared at 4–8 min of chromatogram. The results were expressed as equivalent to an external standard, phorbol-12-myristate-13-acetate. Measurement was performed by the external standart calibration method. MS/MS was performed equipped with ESI source, the detection limit for phorbol esters quantification was 0.01 mg/g.

2.10 Analysis of fatty acid composition.

The methyl esters were prepared from the total lipids separated from hot aqueous extract of kernel (pHs 5, 7 and 9) and callus extract (pH 5) by the method of AOAC (1990). These fatty acid methyl esters were analyzed by a gas chromatography coupled with a mass spectrometry detector (GC-MS, Varian brand), model 3800 CP/Saturn 2000 equipped with the CP-Sil CB 8 column (30 m X 0.25 mm). The initial temperature was 60º C with an elevation rate of 3° C/min until 250 ºC. The split was 1/200. The scanning range was from 30 to 500 m/z. The identification was carried out in comparison with the NIST 98 MS Library (Varian 1998) in addition to the specific literature (Adams 2007). The linear retention index and the theoretical retention confirmed the identifications. A standard fatty acid methyl ester mixture was run and the retention times were used to identify the sample peaks. Fatty acid levels were estimated as area percent of total peak area of methyl esters.

2.11 Statistical analysis

The LC_{50} (lethal concentration, 50%) and 95% confidence intervals (CI_{95}) were calculated by Trimmed Spearman-Karber method by using TSK software version 1.5 (Usepa, 1990).

2.12 *In silico* screening of anthelmintic compounds

The bioactivities of the phytochemicals found in the different samples were predicted by PASS (Prediction of activity spectra for substances) (i.e. an *in silico* tool for prediction of bioactivity) available at (http://www.pharmaexpert.ru/passonline/). The results of PASS software are showed as a value of probability to be active (Pa). The software PASS can not evaluate biocompounds with less of 3 carbon atoms, therefore, the formic acid, acetic acid and ethanol were not predicted their bioactivity (Poroikov and Filimonov, 2001). In order to validate the nematicidal activity of compounds found in the sample, we choice only Pa values above of 0.80.

3 Results and Discussion

3.1 Anthelmintic activity of kernel and callus extracts

The anthelmintic activity was observed in all the hot aqueous extracts of kernels independent of the pH used for extraction, however, the most effective pH extract was 5, followed of 7 and 9, presenting LC_{50} of 16.76, 24.02 and 30.02 $mg.mL^{-1}$ (Table 2). Prior studies have made seed extraction using cold water and disconsidering the pH importance for extraction (Joymati et al., 1998; Eguale and Giday, 2009; Salles et al., 2014), these studies were carried out using *Meloidogyne incognita* (Joymati et al., 1998) and *Haemonchus contortus* (Eguale and Giday, 2009; Salles et al., 2014).

The callus tissue also demonstrated nematicidal activity with a LC_{50} of 14.52 $mg.mL^{-1}$ (Table 3), these callus was 115 days old, with corresponding at deceleration phase in callus growth kinetics (i.e. when the higher yield of callus biomass is obtained) (Costa et al., 2015). The LC_{50} of hot aqueous extract of kernel in pH 5 was again established and reached a LC_{50} of 12.44 $mg.mL^{-1}$ (Table 3), considering that this kernel was prevenient from other harvest (2015).

This result has demonstrated a variation among the LC_{50} established of kernels obtained from different harvests. The nematicidal activity of callus and kernels are similar, nevertheless, kernel can show variation in its nematicidal activiy. This variation in LC_{50} found in kernel extracts can be due to the genetic and physiologic differences even into the same physic nut population (Table 3).

Table 2. Lethal concentration (LC_{50}) of hot aqueous extracts of physic nut (*Jatropha curcas* L.) kernels on free-living nematode *Caenorhabditis elegans* after 24 hours. LC_{50} was estimated using trimmed Spearman-Karber.

Extract	Concentration ($mg.mL^{-1}$)	Mortality (%)	LC_{50}Values ($mg.mL^{-1}$)	Limits 95% confidence ($mg.mL^{-1}$)	TSK Trim value (%)
Hot aqueous (pH 5)	0	0	16.76	14.23-19.73	0.00
	5	5			
	10	25			
	15	26.6			
	20	68.4			
	30	88.2			
	40	100			
Hot aqueous (pH 7)	0	0	24.02	20.10-28.72	14.29
	5	0			
	10	0			
	15	6.25			
	20	46.6			
	30	60.0			
	40	85.7			
Hot aqueous (pH 9)	0	0	30.02	26.74-33.69	12.06
	5	0			
	10	0			
	15	0			
	20	5.3			
	30	50.0			
	40	88.2			

Table 3. Lethal concentration (LC_{50}) of hot aqueous extracts (pH 5) of physic nut (*Jatropha curcas* L.) kernel (harvest in December, 2015) and callus obtained from *in vitro* culture (at 115 days) on free-living nematode *Caenorhabditis elegans* after 24 hours. LC_{50} was estimated using trimmed Spearman-Karber.

Extract	Concentration ($mg.mL^{-1}$)	Mortality (%)	LC_{50}Values ($mg.mL^{-1}$)	Limits 95% confidence ($mg.mL^{-1}$)	TSK Trim value (%)
Kernel (pH 5)	0	0	12.44	10.46-14.79	3.77
	5	8.2			
	10	29.4			
	20	78.6			
	40	100			
	80	100			
Callus (pH 5)	0	0	14.52	12.22-17.26	0.00
	5	13.1			
	10	25.4			
	20	73.5			
	40	94.3			
	80	100.0			

3.2 Cytotoxicity.

In cytotoxicity test by using brine shrimp (*Artemia salina*) the extracts not produced cell toxicity. The hot aqueous extracts (pH5) kernel and callus (115 days of the culture) against *Artemia salina* was not produced mortality, Lethal concentration

50% (LC_{50}) at the concentration >1000 µg.mL^{-1}, these results open new possibilities for application in industries. These results were different, which aqueous extract of *Euphorbia hirta* leaves, the same family, were producted 50% mortality (LC_{50}) at the concentration 80 µg.mL^{-1}.

3.3 Extract characterization by FTIR

Infrared spectra showed that the chemical composition of callus is very different than the kernel (Fig. 7 and Table 4). The infrared spectra of the hot aqueous extracts of *J. curcas* callus, demonstrated the absence of the bands related to asymmetric stretching and symmetrical of the CH_2 groups and aliphatic CH_3 (3000-2930 cm^{-1}). Another notable difference is also in the region of carbonyl groups (C=O), callus extract exhibits band at 1762 cm^{-1} and the kernel extracts is in this band 1749 cm^{-1}, probably due to the first band is a carboxylic acid e the second band is a ketone.

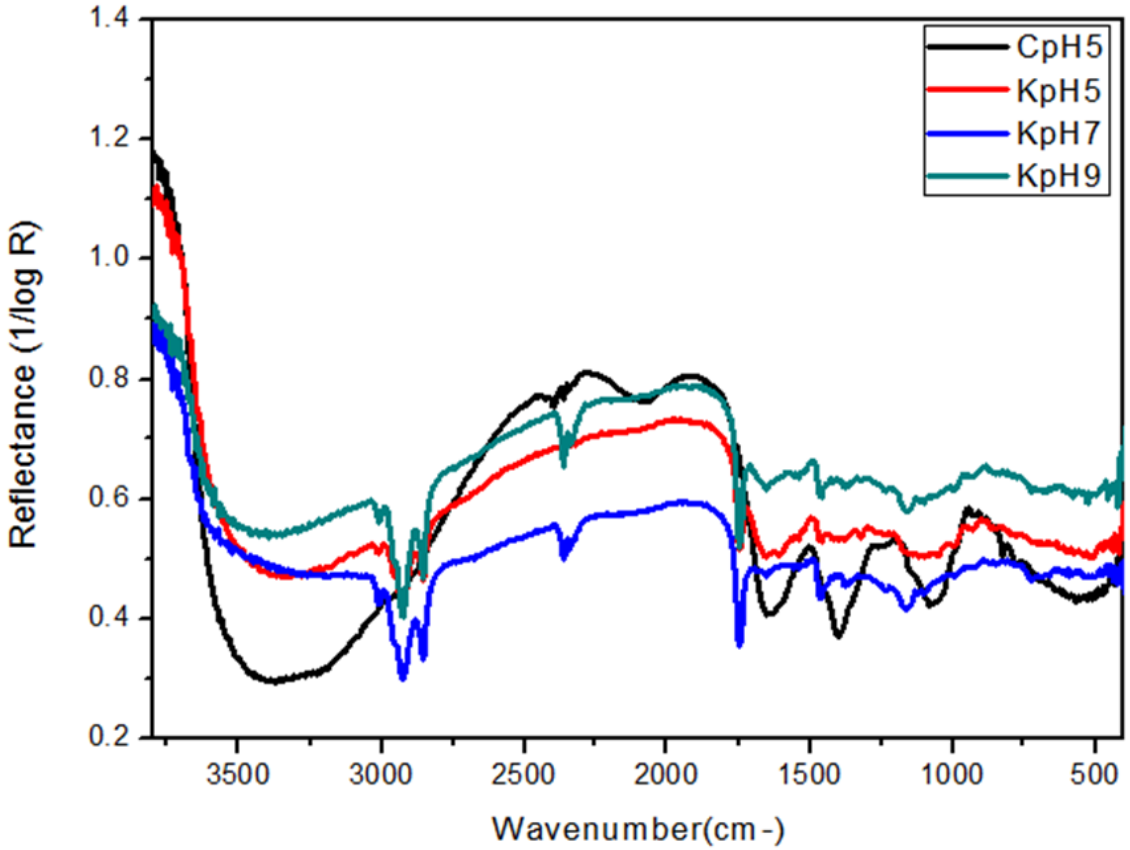

Figure 7 – Reflectance spectra of infrared of the hot aqueous extracts of *Jatropha curcas* kernel and callus in different pH. CpH5 – Extract of callus tissue in pH 5, KpH5 - Extract of kernel in pH 5, KpH7 - Extract of kernel in pH 7 and KpH9 - Extract of kernel in pH 9.

Thamizhselv et al. (2013) reported that seed of *J. curcas* exhibits absorption band around 3010-2930 cm^{-1} corresponding to C-H vibrations stretching of the CH_2 groups due the presence of amino acids and fats, this band may also be characteristic of the presence of aliphatic C-H groups in these compounds. The band in 1750 cm^{-1} (C=O) stretching indicates the presence of carbonyl groups.

Table 4. Bands and Assignments FTIR for *Jatropha curcas* hot aqueous extracts of kernel and callus at different pH. CpH5 – Extract of callus tissue in pH 5, KpH5 - Extract of kernel in pH 5, KpH7 - Extract of kernel in pH 7 and KpH9 - Extract of kernel in pH 9.

Bands				**Assignments**
CpH 5	**KpH 5**	**KpH 7**	**KpH 9**	
3380 *s*	3380 w	-	3380 m	Stretching vibrations O-H or N-H
-	3014 w	3014 w	3014 m	Stretching vibrations CH (aromatics)
-	2956 w	2956 w	2956 w	Stretching vibrations CH (aliphatic)
-	2930 s	2930 s	2930 s	Stretching vibrations (symmetric and assymmetric) CH_2 (aliphatic)
-	2856 s	2856 s	2856 s	Stretching vibrations CH_3 (aliphatic)
2067 *m*	-	-	-	Stretching vibrations C=C (aromatic ring)
1762 w	1749 s	1749 s	1749 s	Stretching vibrations C=O (ketone and carboxilic acid)
-	-	1685 w	-	Stretching vibrations C=C
1650s	1654 w	1652 w	1656 w	Stretching vibrations C=C
-	1544 w	1560 w	1560 w	Stretching NH
-	-	1540 w	1540 w	C=C
1458 w	1458 m	1458 w	1460 w	Stretching CH
	1422 w	1422 w	1422 w	Assymmetric Stretching CH_3
1400 s	1400 w	1400 w	1400 w	In-plane bending vibration, O-H
-	1379 w	1379 w	1379 w	In-plane bending vibration, O-H
-	1322m	-	-	Stretching C-O
1244 w	1240 w	1236 w	1240 w	In-plane bending vibration CH
1078 w	1141 w	1164 w	1164 w	Stretching Coupled C-O + C-C
-	-	-	1124 w	Stretching C-O
-	1100 w	1100 w	1100 w	Stretching C-O
-	1055 w	-	-	Assymmetric vibrations C-O
-	997 m	995 w	999 w	Bending C-H
920 w	930 m	930	920 w	Bending =C-H
896 w	-	-	-	Rocking vibrations, CH_3
864 w	-	844 w	-	Bending =C-H
830 m	-	-	830 w	Bending =C-H
-	780 w	780 w	-	C-H
-	-	-	730 w	Out-of-plane bending , OH
-	630 w	630 w	-	C-H

The present investigations show the presence of carbohydrates, amino acids, amides, esters, ethers, phenols, proteins and fat probably in different amounts of samples of the plants investigated.

3.4 Partial characterization of the extracts

There are very differences among the chemical profile found in the four extracts studied (Table 5 and 6). These chemical profile is corroborated by FTIR analysis already discussed in the previous section.

Table 5. Compounds identified in different hot aqueous extracts of kernel and callus of physic nut (*Jatropha curcas* L.).

	Kernel extract			**Callus extract**
	mg.g^{-1} dry weigth			
Compound	**pH 5**	**pH 7**	**pH 9**	**pH 5**
Cellobiose	nd[1]	0.0163	0.0178	0.009
Glucuronic acid	nd	nd	nd	nd
Galacturonic acid	nd	nd	nd	nd
Gluconic acid	nd	nd	nd	nd
Glucose	0.3524	0.0632	0.2735	0.033
Mannose	nd	nd	nd	nd
Xylose	0.1567	nd	0.1106	0.035
Arabinose	nd	nd	nd	nd
Xylitol	nd	0.0024	0.0022	nd
Furfurilic acid	nd	nd	nd	nd
Glycerol	nd	nd	nd	nd
Formic acid	nd	nd	0.4477	nd
Acetic acid	nd	nd	0.0369	nd
Levulinic acid	nd	nd	nd	nd
Ethanol	nd	nd	0.0384	nd
Hydroxymethylfurfural	nd	nd	nd	nd
Furfural	0.0523	nd	0.0422	nd
Phorbol esters	0.0351	0.0374	0.0388	0.0450

These differences in chemical profile main involving the extraction in different pHs, what influences in high or minor solubility of these compounds according with their chemical groups. The fatty acids are not soluble in water, but their presence in water extracts can be due to hydrolysis of oil during the hot extraction.

The yields for the extracts were 1.3% and 0.61% for kernel and callus extracts (pH 5), 1.2% for kernel extract (pH 7) and 1.18% for kernel extract (pH 9). The spectrometric profile of phorbol esters presented five fragment sizes, being 114.8, 150.8, 230.9, 300.9 and 324.9, corroboring with the data found by Haas et al. (2002).

The nematicidal activity of the furfural is already known, but the LC_{50} of furfural was not established yet against *C. elegans*. However, furfural application is against phytonematoids (El-Mougy et al., 2008). Although, only the kernel extract (pH 5) showed a low concentration of furfural (Table 5). The presence of the furfural in the kernel extract (pH 5) can be an explanation for this extract presented the best performance. The mode of action of furfural against nematodes has been described as the destruction of the nematode cuticle (Burger, 2005). Although, we must consider also the presence of other nematicidal compound did not identified yet.

Table 6. Fatty acids identified in crude oil found in different hot aqueous extracts of kernels and callus of physic nut (*Jatropha curcas* L.).

	Kernel extract			**Callus extract**
Oil yield from extract (%)	12.59	12.80	6.46	0.0
	µg per mg of fatty acids			
Capric acid (C10:0)	nd[1]	2.59	nd	nd
Palmitoleic acid (C16:1)	nd	7.64	4.88	nd
Palmitic acid (C16:0)	216.85	139.72	236.55	nd
Linoleic acid (C18:2)	242.98	347.94	153.86	nd
Oleic acid (C18:1)	300.1	430.17	361.92	nd
Stearic acid (C18:0)	108.08	71.94	123.08	nd
Arachidic acid (C20:0)	nd	nd	119.71	nd

[1]Not detected

Some fatty acids and their derivatives have been reported to have nematicidal activity (Tarjan and Cheo, 1956; Sayre et al., 1965; Djian et al., 1994; Davies et al., 1997). Tarjan and Cheo (1956) reported that straight-chain fatty acid radicals of intermediate carbon-chain length (C8-C10) had the highest nematicidal activity of the fatty acids tested, and that better emulsions would improve nematicidal activity. Djian et al. (1994) have indicated that the nematicidal activity of dicarboxylic fatty acids could be improved by esterification of these compounds.

The nematicial activity of the fatty acids can be described according their LC_{50} 18h against *C. elegans*, being the order of the most actives the linoleic acid (LC_{50} = 5 µg.mL^{-1}), followed by palmitoleic acid (LC_{50} = 10 µg.mL^{-1}), oleic and palmitic acid (LC_{50} = 25 µg.mL^{-1}), stearic acid and decanoic acid or capric acid (LC_{50} = 50 µg.mL^{-1}) and arachidic acid (LC_{50} > 100 µg.mL^{-1}) (Stadler et al., 1994).

The mode of action of fatty acids is yet unknown, however, it was suggested that the probable mechanism of action of the methyl pelargonate (fatty acid) is due to its

physical properties might promote some type of detergent (solubilization) effect that may adversely interfere with the integrity and permeability of the nematode cuticle or hypodermis. Jalal and Read (1983) suggested that the inhibitory effects of fatty acids of intermediate chain length that have been observed in biological systems may involve a direct interaction between the fatty acids and lipophilic regions of the target plasma membranes.

The fatty acid profile was different in kernel extracts in different pHs. The extract obtained in pH 5 does not have the capric acid, palmitoleic acid and arachidic acid, the extract obtained in pH 7 does not have the arachidic acid and the extract obtained in pH 9 does not have the capric acid. The callus extracts did not present these fatty acids (Table 6). The nematicidal activity of the kernel extracts could be increased if these extracts were microemulsioned, whereas fatty acids are not soluble in water. The fatty acids concentrations found in the kernel extracts are above than LC_{50} established for *C. elegans* described by Stadler et al. (1994). However, there is no information about the resistance of this nematode strain in order to compare with the namtode strain used in the present study.

3.5 *In silico* analysis for nematicidal activity of compounds

The biological activity of the compounds found in the hot aqueous extracts, evaluated by *in silico* analysis through prediction of activity spectra for substances (PASS) software, indicated that thirteen phytochemicals were expected to show anthelmintic activity at various levels. *In silico* studies showed that the values of Pa (probability to be active) for these biocompounds found and they varied from 0.436 and 0.865, respectively (Table 7).

Table 7. *In silico* analysis for nematicidal activity of compounds found in all the hot aqueous extracts of *J. curcas* seed and callus by PASS prediction (Filimonov et al., 2014).

Compound	Predicted Activity	Pa*	Extract
Cellobiose	Anthelmintic (nematicidal)	0.681	pH 7 and 9
Glucose	Anthelmintic (nematicidal)	0.660	pH 5, 7 and 9
Xylose	Anthelmintic (nematicidal)	0.437	pH 5 and 9
Xylitol	Anthelmintic (nematicidal)	0.460	pH 7 and 9
Furfural	Anthelmintic (nematicidal)	0.436	pH 5 and 9
Capric acid	Anthelmintic (nematicidal)	0.697	pH 7
Palmitoleic acid	Anthelmintic (nematicidal)	0.626	pH 7 and 9
Palmitic acid	Anthelmintic (nematicidal)	0.697	pH 5, 7 and 9
Linoleic acid	Anthelmintic (nematicidal)	0.580	pH 5, 7 and 9
Oleic acid	Anthelmintic (nematicidal)	0.626	pH 5, 7 and 9
Stearic acid	Anthelmintic (nematicidal)	0.697	pH 5, 7 and 9
Arachidonic acid	Anthelmintic (nematicidal)	0.580	pH 9
TPA**	Anthelmintic (nematicidal)	0.865	-
12-deoxi-16-hydroxiphorbol	Anthelmintic (nematicidal)	0.749	Nd***

*Pa = Probability to be active. ** 12-O-tetradecanoylphorbol-13-acetate (compound does not produced in *J. curcas*, this compound was used as an external standart due to high similarity with PE from *J. curcas*
*** Not determined due to absence of availability of a commercial standard.

Some these compounds with predicted anthelmintic activity already reported in the literature, agreeing with this prediction, being some fatty acids (capric acid, arachidic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid and stearic acid) and the heterocyclic aldehyde furfural (Table 5 and 7). However, the nematicidal activity of cellobiose, xylose, glucose, phorbol esters and xylose were not found in the literature. Although, the glucose shortens the life span of *C. elegans* by downregulating DAF-16/FOXO activity and aquaporin gene expression (Lee et al., 2009). These predicted compounds, but it not described in the literature for nematicidal activity, it is necessary to validate it for this activity.

Phorbol esters are not considered as nematicidal compound in the literature abour physic nut. However, in studies with the species, *Aleurites cordata*, *Aleurites fordii* and *Sapium japponicum* (methanolic extract) of Euphorbiaceae against the pathogenic nematode *Bursaphelenchus lignicolous*, was suggested the activity of these euphorbiaceous plants could to be due to phorbol esters, and their related compounds, odoracin and odoratrin, that showed nematicidal activity (Kawazu et al., 1980).

The TPA presented a LC_{50} = 0.67 $mg.mL^{-1}$ against *C. elegans*, however, TPA is not found in physic nut, but it is used usually as an external standard to determine the phorbol esters concentrations equivalent to TPA (Makkar et al. 1998; He et al. 2011), TPA is very similar with the phorbol ester found in physic nut and is the only available

commercial standard. None phorbol ester commercial internal standard is available for physic nut at the moment.

The TPA LC_{50} was validate in the present study due to have a high Pa value (0.865), this value is larger than the Pa value (0.749) found for 12-deoxi-16-hydroxiphorbol (Table 7), the six phorbol ester of *J. curcas* are all derivatives from 12-deoxi-16-hydroxiphorbol, nevertheless, all these six intramolecular diterpene esters are unstable (Haas et al., 2002). We can consider the nematicidal activity of TPA as moderate comparing with other LC_{50} available in the literature, this result suggests the nematicidal activity of phorbol esters of *J. curcas* is also moderate. Other question is about the concentrations observed in these extracts, varying from 0.0351 to 0.045 $mg.mL^{-1}$ (Table 5), what represents approximately 17 times minor than the LC_{50} established for TPA (Table 8). The callus extract had obtained the major concentration of phorbol ester, 0.045 $mg.mL^{-1}$ (Table 5).

Table 8. Lethal concentration (LC_{50}) of 12-O-tetradecanoylphorbol-13-acetate (TPA) on free-living nematode *Caenorhabditis elegans* after 24 hours. LC_{50} was estimated using trimmed Spearman-Karber.

Concentration ($mg.mL^{-1}$)	**Mortality (%)**	**LC_{50}Values ($mg.mL^{-1}$)**	**Limits 95% confidence ($mg.mL^{-1}$)**	**TSK Trim value (%)**
0	0	0.67	0.59-0.77	15.25
0.25	5.8			
0.50	22.2			
1.00	84.7			

In another study, *J. curcas* calli produced phorbol esters varying from 2.44 to 5.27 mg/g dry weight, these calli were induced from seed explants that were cultured in MS medium supplemented with 2.5 $mg.L^{-1}$ NAA (naphtalene acetic acid) and 0.5 $mg.L^{-1}$ BAP (benzyl amino purine) (Wirasutisna et al., 2011). However, in this another study, phorbol esters were extract with dichloromethane, so solubilized with ethanol and in this present study, we had extracted with hot water.

Phorbol esteres are purgative, skin-irritant and, of more concern, are tumour-promoters. Phorbol esteres act as a structural analogue of diacylglycerol, an activator of protein kinase C (PKC) (Zhang et al., 1995). Unlike diacylglycerol, which has a short biological half-life, the activation of PKC by phorbol esteres has a prolonged effect which leads to a number of biological activities, including the triggering of cell proliferation (Goel et al., 2007). Moreover, phorbol esters showed a molluscicidal

(Devappa et al., 2012), insecticidal (Jing et al., 2004), and fungicidal activities (Devappa et al., 2012). Now, these compounds can be exploited also as anthelmintics.

4 CONCLUSIONS

How aqueous extracts of kernels and calli of *J. curcas* have anthelmintic activity. Extraction in different pH presented different results. The most effective pH extract was 5, followed of 7 and 9, presenting LC_{50} (lethal concentration, 50%) of 16.76, 24.02 and 30.02 $mg.mL^{-1}$. Hot aqueous extract (pH 5) obtained from callus tissue (with 115 days of culture) presented a LC_{50} of 14.52 $mg.mL^{-1}$. The anthelmintic activity of callus and kernel are similar, however, kernel activity can vary due to harvest and genetic variability. These extracts were not toxic against *Artemia salina*. The nematicidal fitochemicals were not completed elucidated, but different pH extraction and tissue type presented different nematicidal compounds, such as fatty acids, furfural and phorbol esters.

5 GENERAL CONCLUSIONS

The hypocotyl explants from seedlings allowed the *in vitro* production of callus growth of *Jatropha curcas* L.

The kinetics of the callus growth curve of *Jatropha curcas* L. exhibited a sigmoid standard curve and obtained at the 120th day of culture the highest percentage of oil content (extract ethereal).

The lipid profile obtained in callus oil was different from the seed oil profile.

The anthelmintic activity of callus and kernel are similar, but were not completed elucidated type presented nematicidal compounds.

6 SUGGESTIONS FOR FUTURE RESEARCH

- Development of a continuous process from callus cultures of *Jatropha curcas* as a profitable source for scaling up secondary metabolite production;
- Molecular characterization of callus curve to identify the genetic material of the *Jatropha curcas*;
- These results could be used in further research to establish *in vitro* cell suspension, hairy root cultures can promote a higher production of biomass and increase secondary metabolits production which could be a prospective for the pharmaceutical industry.
- To identify. characterize and evaluate the effect of different extraction solvents of a new bioactive compounds in *Jatropha curcas* and to develop nanostrutures with anthelmintic activity;

7 PAPERS PUBLISHED

BIER, M. C. J.; MEDEIROS, A. B. P. ; OLIVEIRA, J. S.; CÔCCO, L C.; COSTA, J. L.; CARVALHO, J. C.; SOCCOL, C. R. Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes. **The Journal of Supercritical Fluids**, v. 110, p. 97-102, 2016.

HABIBI, P.; SA, M. F. G.; Silva, A. L. L.; MAKHZOUM, A.; COSTA, J. L.; BORGHETTI, I. A.; SOCCOL, C. R. Efficient genetic transformation and regeneration system from hairy root of Origanum vulgare. **Physiology and Molecular Biology of Plants**, v. 22, p. 271-277, 2016.

GOLLO, A. L.; SILVA, A. L. L.; LIMA, K. K. D.; COSTA, J. L.; CAMARA, M. C.; BIASI, L. A.; RODRIGUEZ, C.; VANDENBERGUE, L. P. S.; THOMAZ-SOCCOL, V.; SOCCOL, C. R. Developing a plant culture medium composed of vinasse originating from Haematococcus pluvialis culture. **Pakistan Journal of Botany**, v. 48, p. 295-303, 2016.

SILVA, A. L. L.; GOLLO, A. L.; BRONDANI, G. E.; HORBACH, M. A.; OLIVEIRA, L. S.; MACHADO, M. P.; LIMA, K. K. D.; COSTA, J. L. Micropropagation of Eucalyptus saligna SM. from cotyledonary nodes. **Pakistan Journal of Botany**, v. 47, p. 311-318, 2015.

CHAGAS-JUNIOR, A. F.; OLIVEIRA, A. G.; OLIVEIRA, L. A.; SANTOS, G. R.; CHAGAS, L. F. B.; SILVA, A. L. L.; COSTA, J. L. Production of indole-3-acetic acid by Bacillus isolated from different soils. **Bulgarian Journal of Agricultural Science**, v. 21, p. 282-287, 2015.

COSTA, J. L.; SILVA, A. L. L.; BIER, M. C. J.; BRONDANI, G. E.; GOLLO, A. L.; Letti, L. A. J.; ERASMO, E. A. L.; SOCCOL, C. R. Callus growth kinetics of physic nut (Jatropha curcas L.) and content of fatty acids from crude oil obtained in vitro. **Applied Biochemistry and Biotechnology**, v. 176, p. 892-902, 2015.

SILVA, A. L. L.; COSTA, J. L.; GOLLO, A. L.; SANTOS, J. D.; BIASI, L. A.; CARVALHO, J. C.; SOCCOL, C. R. Development of a vinasse culture medium for plant tissue culture. **Pakistan Journal of Botany**, v. 46, p. 2195-2202, 2014.

COSTA, J. L.; SILVA, A. L. L.; CHAGAS-JUNIOR, A. F.; SCHEIDT, G. N.; VERAS-JÚNIOR, R. V. Organogenesis in zygotic embryos and hypocotyls of physic nut (Jatropha curcas L.) under 6-benzylaminopurine levels. **Advances in Forestry Science**, v. 1, p. 71-73, 2014.

SILVA, A. L. L.; OLIVEIRA, Y.; PROCOPIUK, M.; Mudry, C. S.; BRONDANI, G. E.; COSTA, J. L.; SCHEIDT, G. N. Expressão transiente do gene uidA em explantes foliares de Eucalyptus saligna Sm. transformado via Agrobacterium tumefaciens. **Bioscience Journal** (UFU. Impresso), v. 29, p. 1-7, 2013.

SANTOS, J. D.; SILVA, A. L. L.; COSTA, J. L.; SCHEIDT, G. N.; NOVAK, A. C.; SYDNEY, E. B.; SOCCOL, C. R. Development of a vinasse nutritive solution for hydroponics. **Journal of Environmental Management**, v. 114, p. 8-12, 2013.

SILVA, A. L. L.; RODRIGUES, C.; COSTA, J. L.; MACHADO, M. P.; PENHA, R. O.; BIASI, L. A.; VANDENBERGHE, L. P. S.; SOCCOL, C. R. Gibberellic acid fermented extract obtained by solid-state fermentation using citric pulp by Fusarium moniliforme: influence on Lavandula angustifolia mill. cultivated in vitro. **Pakistan Journal of Botany**, v. 45, p. 2057-2064, 2013.

REFERENCES

ADAMS, R. P. **Identification of essential oil components by gas chromatography/mass spectrometry**. 4th. Carol Stream: Allured Publishing Corporation, 2007.

AOAC. **Official methods of analysis of the Association of Official Analytical Chemists 15th**. Washington: Association of Official Analytical Chemists, 1990.

BERG, AJ, HORSTEN, SF, KETTENES VAN DEN BOSCH, JJ, KROES, BH, BEUKELMAN, CJ, LOEFLANG, BR, LABADIE, RP (1995) Curcacycline A: a novel cyclic octapeptide isolated from the latex of Jatropha curcas Linn. FEBS Letters 358: pp. 215-218

BLIGH, E. G.; DYER, W. J. A rapid method of total lipid extraction and purification. **Canadian journal of biochemistry and physiology**, v. 37, n. 8, p. 911-917, 1959.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: pp. 248-254, 1976.

BURGER, G. An overview of the development of furfural (Crop Guard®) as a nematicide in South Africa: **The chemistry of furfural within the soil and agricultural environment**. Proc. Annu. Int. Res. Conf. on Methyl Bromide Altern. and Emiss. Reduct, 2005. 1 p.

CARVALHO, D. C.; SILVA, A. L. L.; SCHUCK, M. R.; PURCINO, M.; TANNO, G. N.; BIASI, L. A. Fox grape cv. Bordô (*Vitis labrusca* L.) and grapevine cv. Chardonnay (*Vitis vinifera* L.) cultivated in vitro under different carbohydrates, amino acids and 6-benzylaminopurine levels. Brazilian Archives of Biology and Technology 56: pp. 191-201, 2013.

CHABRA, S. C.; MAHUNNAH, R. L. A.; MSHIU, E. N. Plants used in traditional medicine in Eastern Tanzania. III. Angiosperms (Euphorbiaceae to Menispermaceae). **Journal of Ethnopharmacology**, v. 28, n. 3, p. 255-283, 1990.

COSTA, J. L.; LIMA, R. P.; SILVA, A. L. L.; SCHEIDT, G. N.; ERASMO, E. A. L. Initial growth of plants of physic nut under shading in the Gurupi county, Tocantins State, Brazil. **Journal of Biotechnology and Biodiversity**, v. 2, n. 4, p. 43-47, 2011.

COSTA, J. L. SILVA, A. L. L.; BIER, M. C. J.; BRONDANI, G. E.; GOLLO, A. L.; LETTI, L. A. J.; ERASMO, E. A. L.; SOCCOL, C. R. Callus growth kinetics of physic nut (*Jatropha curcas* L.) and content of fatty acids from crude oil obtained *in vitro*. **Applied biochemistry and biotechnology**, v. 176, n. 3, p. 892-902, 2015.

COSTA, J. L.; SILVA, A. L. L.; SCHEIDT, G. N.; ERASMO, E. A. L.; SOCCOL, C. R. *In vitro* establishment of seeds of physic nut (*Jatropha curcas* L.) - Euphorbiaceae. **Caderno de Pesquisa Série Biologia**, v. 22, n. 3, p. 5-12, 2010.

CORREA, S. M.; ATEHORTÚA, L. Lipid profile of in vitro oil produced through cell culture of Jatropha curcas L. Journal of AOAC International 95: pp. 1161-1169, 2012.

DAVIES, E. L.; MEYERS, D. M.; DULLUM, C. J.; FEITELSON, J. S. Nematicidal activity of fatty acid esters on soybean cyst and root-knot nematodes. **Journal of nematology**, v. 29, n. 4S, p. 677-684, 1997.

DEVAPPA, R. K.; RAJESH, S. K.; KUMAR, V.; MAKKAR, H. P. S.; BECKER, K. Activities of *Jatropha curcas* phorbol esters in various bioassays. **Ecotoxicology and environmental safety**, v. 78, p. 57-62, 2012.

DJIAN, C.; PONCHET, M.; CAYROL, J. Nematocidal properties of carboxylic-acids and derivatives. **Pesticide biochemistry and physiology**, v. 50, n. 3, p. 229-239, 1994.

EGUALE, T.; GIDAY, M. *In vitro* anthelmintic activity of three medicinal plants against *Haemonchus contortus*. **International Journal of Green Pharmacy**, v. 3, n. 1, p. 29-34, 2009.

EL-MOUGY, N. El-Gamal, N. G.; Mohamed, M. M. M.; Abdel-Kader, M. M. Furfural approaches as control measures against root rot and root-knot incidence of tomato under greenhouse and field conditions. **Journal of Plant Protection Research**, v. 48, n. 1, p. 93-105, 2008.

GOEL, G. MAKKAR, H. P. S.; FRANCIS, G.; BECKER, K. Phorbol esters: structure, biological activity, and toxicity in animals. **International journal of toxicology**, v. 26, n. 4, p. 279-288, 2007. ISSN 1091-5818.

GOLLO, A. L. SILVA, A. L. L.; LIMA, K. K. D.; COSTA, J. L.; CAMARA, M. C.; BIASI, L. A.; RODRIGUES, C.; VANDENBERGHE, L. P. S.; SOCCOL, V. T.; SOCCOL, C. R. Developing a plant culture medium composed of vinasse originating from *Haematococcus pluvialis* culture. **Pakistan Journal of Botany**, v. 48, n. 1, p. 295-303, 2016.

HAAS, W.; STERK, H.; MITTELBACH, M. Novel 12-Deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. **Journal of Natural Products**, v. 65, n. 10, p. 1434-1440, 2002.

HABIBI, P. SA, M. F. G.; SILVA, A. L. L.; MAKHZOUM, A.; COSTA, J. L.; BORGHETTI, I. A.; SOCCOL, C. R. Efficient genetic transformation and regeneration system from hairy root of *Origanum vulgare*. **Physiology and Molecular Biology of Plants**, v. 22, n. 2, p. 271-277, 2016.

HALDER, T.; GADGIL, V. N. Comparison of fatty acid patterns in plant parts and respective callus cultures of Cucumis melo. Phytochemistry 23: pp. 1790-1791, 1984.

HE, W. KING, A. J.; KHAN, M. A.; CUEVAS, J. A.; RAMIARAMANANAC, D.; GRAHAM, I. A. Analysis of seed phorbol-ester and curcin content together with genetic diversity in multiple provenances of *Jatropha curcas* L. from Madagascar and Mexico. **Plant physiology and biochemistry**, v. 49, n. 10, p. 1183-1190, 2011.

HELLER, J. Physic nut. Jatropha curcas L. **Promoting the conservation and use of underutilized and neglected crops**. International Plant Genetic Resources Institute, Rome, 1996.

HORBACH, M. A.; MALAVASI, U. C.; MALAVASI, M. M.; AJALA, M. C.; LIMA, P. R.; SCHULZ, D. G. Propagation methods for physic nut (*Jatropha curcas*). **Advances in Forestry Science**, v. 1, n. 1, p. 53-57, 2014.

JAIN, M. N.; JAIN, S. R. Therapeutic activity of Ocimum basilicum var. album, **Planta Medica**, v. 22, p.66-70, 1972.

JALAL, M.; READ, D. The organic acid composition of Calluna heathland soil with special reference to phyto-and fungitoxicity. **Plant and Soil**, v. 70, n. 2, p. 257-272, 1983.

JING, L.; FANG, Y.; FEN-HONG, W.; BI-SONG, Y.; FANG, C. Insecticidal activity of extracts from *Jatropha curcas* seed against *Lipaphis erysimi*. **Acta Phytophylacica Sinica**, v. 31, n. 3, p. 289-293, 2004.

JOYMATI, L.; DHANACHAND, C.; DEVI, L. S. Effect of plants extracts on *Meloidogyne incognita*. **Indian Journal of Nematology**, v. 28, n. 2, p. 225-230, 1998.

KALAIARASAN, P. SENTHAMARAI, M.; RAMESH, D.; SUDHEER, M. J. Jatropha: an efficient organic amendment for the management of root-knot nematode, *Meloidogyne incognita* in tomato. **Indian Journal of Nematology**, v. 37, n. 2, p. 115-118, 2007.

KAWAZU, K. et al. A convenient screening method for nematicidal activity. **Agricultural and Biological Chemistry**, v. 44, n. 3, p. 631-635, 1980.

KUPCHAN, SM.; SIGEL, CW.; MATZ, MJ.; GILMORE, CJ.; BRYAN, RF. Structure and stereochemistry of jatrophone, a novel macrocyclic diterpenoid tumor inhibitor. Journal of the American Chemical Society 98: pp. 2295-2300, 1976.

LI, J, YAN, F, WU, FH, YUE, BS, CHEN, F. Insecticidal activity of extracts from Jatropha curcas seed against Lipaphis erysimi. Acta Physica Sinica 31: pp. 289-293, 2004.

LI, Y. L.; ZHANG, P.; HE, Y. Perspective of the development and application of Jatropha curcas in the dry-hot valley of Panzhihua. Guangxi Trop Agri. 2: pp. 39-40, 2006.

LIN, J, YAN, F, TANG, L, CHEN, F. Antitumor effects of curcin from seeds of Jatropha curcas. Acta Pharmacologica Sinica 24: pp. 241-246, 2003.

LEE, S.-J.; MURPHY, C. T.; KENYON, C. Glucose shortens the life span of *C. elegans* by downregulating DAF-16/FOXO activity and aquaporin gene expression. **Cell metabolism**, v. 10, n. 5, p. 379-391, 2009.

LÓPEZ-VILLALOBOS, A.; DODDS, P. F.; HORNUNG, R. Lauric acid improves the growth of zygotic coconut (*Cocos nucifera* L.) embryos in vitro. **Plant Cell Tissue Organ Culture,** 106, 317-327, 2011.

MAKHZOUM, A. B. SHARMA, P.; BERNARDS, M. A.; TRÉMOUILLAUX-GUILLER, J. Hairy Roots: An Ideal Platform for Transgenic Plant Production and Other Promising Applications. In: GANG, R. D. (Ed.). **Phytochemicals, Plant Growth, and the Environment**. New York, NY: Springer New York, v.42, 2013. p.95-142.

MAKKAR, H.; ADERIBIGBE, A.; BECKER, K. Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. **Food chemistry**, v. 62, n. 2, p. 207-215, 1998.

MARTÍNEZ-HERRERA, J.; SIDDHURAJU, P.; FRANCIS, G.; DÁVILA-ORTÍZ, G.; BECKER, K. Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. Food Chemistry 96: pp. 80-89, 2006.

MONTEIRO, M. V. B.; BEVILAQUA, C. M.; MORAIS, S.M.; MACHADO, L. K.; CAMURÇA-VASCONCELOS, A. L.; CAMPELLO, C. C.; RIBEIRO, W. L.; MESQUITA, M. A. Anthelmintic activity of *Jatropha curcas* L. seeds on *Haemonchus contortus*. **Veterinary Parasitology**, v. 182, n. 2, p. 259-263, 2011.

MILLER, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Biochemistry 31: pp. 426-428, 1959.

MULLA, W. A.; THORAT, V. S.; PATIL, R. V.; BURADE, K. B. Anthelmintic activity of leaves of Alocasia indica Linn. **International Journal of PharmTechn Research,** v. 2, n. 1, p. 26-30, 2010.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia plantarum**, v. 15, n. 3, p. 473-497, 1962.

NOGUEIRA, R. C.; PAIVA, R.; LIMA, E. C.; SOARES, G. A.; OLIVEIRA, L. M.; SANTOS, B. R.; EMRICH, E. B.; CASTRO, A. H. F. Curva de crescimento e análises bioquímicas de calos de murici-pequeno (*Byrsonima intermedia* A. Juss.). Revista Brasileira de Plantas Medicinais, Botucatu 10: pp. 44-48, 2008.

EMBRAPA. Núcleo Tecnológico para Informática. SOC–Software Científico, Campinas, 1989.

POROIKOV, V.; FILIMONOV, D. Computer-aided prediction of biological activity spectra. Application for finding and optimization of new leads. **Rational Approaches to Drug Design**, p. 403-407, 2001.

REDDY, M. P.; PAMIDIMARRI, D. S. Biology and biotechnological advances in Jatropha curcas - a biodiesel plant. In: (Ramawat K. G. ed.) Desert plants. Springer Berlin Heidelberg. pp. 57-71, 2010.

RONG, F.; WANG, S. H. Identification of curcin by western blot in calli generated from explants of Jatropha curcas L. Journal of Sichuan University (Natural Science Edition) 42: pp. 211-214, 2005.

SALLES, H. O.; BRAGA, A. C. L.; NASCIMENTO, M. T. S. C.; SOUSA, A. M. P.; LIMA, A. R.; VIEIRA, L. S.; CAVALCANTE, A. C. R.; EGITO, A. S.; ANDRADE, L. B. S. Lectin, hemolysin and protease inhibitors in seed fractions with ovicidal activity against *Haemonchus contortus*. **Revista Brasileira de Parasitologia Veterinária**, v. 23, n. 2, p. 136-143, 2014.

SANTOS-FILHO, PR.; SANTOS, B. R.; BARBOSA, S.; VIEIRA, L. R.; FREITAS, N. C.; DIAS, D. F.; SANTOS, M. H. Growth curve, biochemical profile and phytochemical analyses in calli obtained from the procambium segments of Bacupari. **Brazilian Archives of Biology and Technology** 57: pp. 326-333, 2014.

SAYRE, R.; PATRICK, Z.; THORPE, H. Identification of a selective nematicidal component in extracts of plant residues decomposing in soil. **Nematologica**, v. 11, n. 2, p. 263-268, 1965.

SCHEIDT, G. N.; SILVA, A. L. L.; OLIVEIRA, Y.; COSTA, J. L.; BIASI, L. A.; SOCCOL, C. R. *In vitro* growth of *Melaleuca alternifolia* Cheel in bioreactor of immersion by bubbles. **Pakistan Journal of Botany**, v. 43, n. 6, p. 2937-2939, 2011.

SERRA, A. G. P.; PAIVA, R.; PAIVA, P. D. O. Análises bioquímicas de calos formados de explantes foliares de castanha do Brasil (Bertholletia excelsa H.B.K.). Ciencia e Agrotecnologia 24: pp. 833-840, 2000.

SHANKER, C.; DHYANI, S. K. Insect pests of *Jatropha curcas* L. and the potential for their management. **Current Science**, v. 91, n. 2, p. 162-163, 2006.

SILVA, A. L. L.; COSTA, J. L.; GOLLO, A. L.; SANTOS, J. D.; FORNECK, H. R.; BIASI, L. A.; SOCCOL, V. T.; CARVALHO, J. C.; SOCCOL, C. R. Development of a vinasse culture medium for plant tissue culture. **Pakistan Journal of Botany**, v. 46, n. 6, p. 2195-2202, 2014.

SMITH, R. H. Plant tissue culture: techniques and experiments. San Diego: Academic, 1992. 171p.

SOLIS, G. M.; PETRASCHECK, M. Measuring *Caenorhabditis elegans* life span in 96 well microtiter plates. **Journal of Visualized Experiments**, n. 49, p. e2496-e2496, 2011.

SOLIS, P. N. WRIGHT, C. W.; ANDERSON, M. M.; GUPTA, M. P.; PHILLIPSON, J. D. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). **Planta medica**, v. 59, n. 03, p. 250-252, 1993.

SOOMRO, R.; MEMON, R. A. Establishment of callus and suspension culture in *Jatropha curcas*. **Pakistan Journal of Botany**, v. 39, n. 7, p. 2431-2441, 2007.

STADLER, M. MAYER, A.; ANKE, H.; STERNER, O.; Fatty acids and other compounds with nematicidal activity from cultures of Basidiomycetes. **Planta medica**, v. 60, n. 2, p. 128-132, 1994.

STEIN, W.; MOORE, S. A modified ninhydrin reagent for photometric determination of amino acids and related compounds. The Journal of Biological Chemistry 176: pp. 367-372, 1948.

TARJAN, A. C.; CHEO, P. C. **Nematocidal value of some fatty acids**. Kingston: University of Rhode Island, 1956, 41p.

THAMIZHSELVI, R.; SELVARAJAN, P.; RAVI, S. Fourier transform infrared spectral analysis of kernel of physic nut (*Jatropha curcas*). **International Journal of Recent Scientific Research**, v. 4, n. 11, p. 1855-1857, 2013.

USEPA. **Trimmed spearman-karber (TSK) program version 1.5 ecological monitoring research division**. Cincinnati: Environmental Monitoring Systems Laboratory, 1990.

VARIAN. **NIst 98 MS Library Database**. ver. 1.7. EUA: 1998.

WATT, J. M.; BREYER-BRANDWIJK, M. G. **The medicinal and poisonous plants of southern and eastern Africa: being an account of their medicinal and other uses, chemical composition, pharmacological effects and toxicology in man and animal. Michigan**: E. & S. Livingstone, 1962. 1457p.

WEI, Q.; LIAO, Y.; ZHOU, L. J.; ZHOU, J. X.; WANG, S. H, Chen, F. Antifungal activity of curcin from seeds of Jatropha curcas. Chinese Journal of Oil Crop Sciences 26: pp. 71-75, 2004.

WIRASUTISNA, K. R. Reducing of phorbol ester content in callus cultures of physic nut (*Jatropha Curcas* L.) using manganese chloride and n-ethylmaleimid. **Pharmacognosy Journal**, v. 3, n. 20, p. 42-46, 2011.

YEMM, E. W.; WILLIS, A. J. The estimation of carbohydrates in plant extracts by anthrone. The Biochemical Journal 57: pp. 508-514, 1954.

ZHANG, G. KAZANIETZ, M. G.; BLUMBERG, P. M.; HURLEY, J. H. Crystal structure of the Cys2 activator-binding domain of protein kinase Cδ in complex with phorbol ester. **Cell**, v. 81, n. 6, p. 917-924, 1995.

ZHANG, X. The molluscidal activities of different extracts from *Jatropha curcas* L. against Pomacea canaliculata. **Journal of Anhui Agricultural University**, 40: pp. 3349-3350, 2012.